# DICHLOROPHOS MEDIATED HEMATOPOIETIC ORGANS GENOTOXICITY IN *Mus musculus*

## DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
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By

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UNDER THE SUPERVISION OF

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# Certificate

This is to certify that the dissertation entitled **"Dichlorophos mediated hematopoietic organs genotoxicity in *Mus musculus*"** has been carried out by Miss Nazia Nazam, under my supervision in the Molecular Genotoxicity Laboratory, Section of Genetics, Department of Zoology, AMU, Aligarh and submitted in partial fulfillment of the requirement for the award of the degree of Master of Philosophy in Zoology (Genetics), AMU, Aligarh.

Place: Aligarh
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# SELF DECLARATION CERTIFICATE

I hereby declare that the dissertation entitled **"Dichlorophos mediated hematopoietic organs genotoxicity in *Mus musculus*"** submitted to the Aligarh Muslim University, Aligarh, in partial fulfillment of the requirement for the award of the degree of Master of Philosophy in Zoology (Genetics), is the result of research work carried out by me under the supervision and guidance of **Prof. Waseem Ahmad (Faridi)**, Section of Genetics, Department of Zoology, AMU, during the period of 2010 □ 2012.

I further declare that these results have not been submitted for any other degree.

Place: Aligarh

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**Nazia Nazam**

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# List of Abbreviations

| | |
|---|---|
| mg | milligram |
| kg | kilogram |
| w/w | weight by weight |
| v/v | volume/volume |
| mg/kg bwt | milligram per kilogram body weight |
| ml | millitre |
| °C | degree Celsius |
| µ | micron |
| min | minute |
| hr | hour |
| rpm | revolutions per minute |
| $LD_{50}$ | median lethal dose |
| ip | intraperitoneal |
| % | percent |
| SE | standard error |
| DDVP | 2, 2-Dichlorovinyl dimethyl phosphate |

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# INTRODUCTION

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- **Genotoxicity** - An Overview
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- **Candidate Compound Dichlorophos (DDVP)** - Some background information

## ▪ **Genotoxicity** – An Overview

Genotoxicity – an applied science connected directly to human and environmental health – draws on data and methodology from many basic sciences notably physiology, pharmacology, metabolic studies, genetics, embryology and chemistry. This discipline, in the last two decades, appeared as the most important source of data on health effects for chemical safety evaluations on existing and upcoming products. The initial identification of many potentially hazardous chemicals inevitably accomplished through human exposure in most of the cases. The lack of information follows studies through extensive toxicological researches usually done by animal toxicology.

The systemic investigation bring forth the effect of physical, chemical or biological agents exerting themselves on the genetic system of the organism and the subsequent expression of these changes come under the purview of genotoxicity. Genotoxic effects are considered among the most serious side effects of any agent that reacts with nuclear DNA. Consequently, these agents can be distinguished as mutagenic or carcinogenic or both in an organism at sub-toxic concentration. The primary objective of the genotoxic assessment is to provide the outcome by the toxic substances in experimental models especially from genetic point of view and extrapolating the mammalian genetic toxicity test results for human benefits. The data from genotoxicity testing batteries provide an indication of whether or not there is genotoxic activity; and if so, aims at assessing a possible mutagenic mode of action including genetic damage, carcinogenicity and heritable genetic risk. The goals of genotoxicity assessment is achieved with a set of screening tests designed to maximize the chance of detecting genotoxic substances, and not to independently predict carcinogenicity alone (Elespuru et al., 2009).

More specifically, genetic toxicology identifies and analyses the action of agents with toxicity directed towards the hereditary components of living systems. Agents bringing about alterations in nucleic acids and associated components at sub-toxic exposure level, resulting in modified hereditary characteristics or DNA inactivation, are classified as genotoxic. These substances usually have common chemical or physical properties facilitating their interaction with nucleic acids, thereby exerting a toxic effect on the genetic material of cells and

altering the genome of exposed organism. Such substances increase the error rate in the reduplication of the genome and induce mutations by damaging the DNA. The toxicants can damage the genetic complex at certain concentrations besides, produce acute nonspecific cytotoxicity and even death. However, the primary objective of genetic toxicology remains to detect and analyze the hazard potential of those agents that are highly specific genetic interactions at sub – toxic concentrations. Thus, genotoxicity has evolved to play a dual role in safety evaluation programs: the first one being, the implementation of testing mutagenesis and risk assessment methods to define the impact of genotoxic agents found in the environment and secondly, the application of genetic toxicology testing methods to the detection and mechanistic understanding of carcinogenic chemicals. Mutations in the germ cells are passed on to the organism's offspring and can cause congenital or hereditary defects, while similar changes in somatic cells can result in cell death, an increased risk of diseases and even cancer.

Then, there is another use of chemicals, as agents in terms of pesticides and herbicides with an aim to increase the crop productivity. It has aroused a great concern among the environmental and health scientists due to their adverse effects in targets as well as non-target species. Although reasonable information is available regarding their environmental and ecological impact, not much is known with regard to its toxicity in the mammalian test system; more so when, hundreds of pesticides and chemicals continued to be pumped in the environment every year.

Coming to pesticides; these are the most broadly used chemicals in modern day agriculture, and there seems to be not let up in the near future. India is the largest producer all over Asia, and holds a high rank in the world for the use of pesticides (Patel et al., 2007). Statistics reveals that more than half percent of Indian population is occupied in agriculture and is therefore, susceptible to pesticide exposure (Gupta, 2004). These by any means have a widespread use in public health and agricultural programs which has not only led to, but consistently contributing in environmental pollution, possible health hazards and poisoning (Bradberry et al., 2005; Yamashita et al., 1997). As early as 1996, Bro-Rasmussen showed that some of these compounds are either non-biodegradable or have low biodegradability and

tend to be toxic at higher concentrations. Even a low level continuing exposure of these pesticide chemicals is carcinogenic (Zahm and Blair, 1992).

The principle classes of compounds used as pesticides or herbicides are **organophosphates**, organochlorines, carbamates, pyrethroids and certain inorganic compounds as well. The possible adverse health effects related to all the above classes of pesticides; mutagenicity and carcinogenicity are of special significance because of the long dormant period between exposure and effect becoming apparent (Motulsky, 1984; Tezuka et al., 1980). Not only these group of chemicals affect target organisms but more seriously, the side effects such as human and animal poisoning, the familiar pesticide resistance and pest resurgence – had made the scenario more challenging (Pimentel et al., 1991). Given to their ability to react with the nuclear DNA – of which we referred earlier – creates the most serious and probable side effect. Heritable genetic diseases, reproductive dysfunction and birth defects are some to mention besides other possible ill effects due to prolonged exposure to these pesticides. Harmful effects of pesticides are thus serious since all of them contribute finally to near total contamination of the environment, food, feed and fibre and cause disruption of the non-target organism. Their study has been done on many tissues and organs, and yet suitability of organ has to be judged on its sensitivity and target specificity.

- **Relevance of Hematopoietic Organs in Genotoxicity**

The kidney, liver, bone marrow, spleen and blood itself are the major hematopoietic organs involved in the production of factors responsible for the regulation of cellular composition of blood. These hematopoietic tissues are sensitive to external influences, toxicity and neoplastic disorders (NIEHS, 2001; Weiss, 2000). The bone marrow is the important potential target of most of chemical exposure (Lund, 2000). The bone marrow assumes greater significance as not only it is being the major hematopoietic organ but also functions as a primary lymphoid tissue (Picker and Siegelman, 1999). It is predominantly present within the central cavities of axial and longer bones and consists of hematopoietic tissue islands and adipose cells surrounded by vascular sinuses interspersed within a meshwork of trabecular bone. Approximately, 80% of the marrow is reported to be hematopoietic tissue in rats and mice (Valli et al., 2002). Since effects of a compound may be

elicited in the circulating blood cell mass or the production of blood cells – evaluation of single or serial whole blood samples and smears, bone marrow aspirates and study on other hematopoietic tissues like liver, kidney, spleen etc – are vital to understand the alterations that may occur in toxicity studies. The chemical agents may directly interact with the hematopoietic tissue or its metabolite may elicit the toxic response indirectly through injury to other organ systems or metabolic pathways and hence, these cells have been mainly targeted to evaluate cytogenetic and genotoxic response. Assessment of hematopoietic tissue is an essential and routine procedure in the investigation of health effects in toxicology and safety assessment studies. In this case, Dichlorophos – although having wide range of application – has relatively not many studies, that too reporting confusing results, is targeted.

- **Candidate Compound Dichlorophos (DDVP) – Some background information**

The organophosphorus (OP) compounds are among the most favoured insecticide (Tripathi and Srivastav, 2010). These are used worldwide in the control of agricultural, household and veterinary pests as they inhibit a key enzyme in the nervous system called cholinesterase from working, and hence disrupt brains and nervous systems.

Being one of the most important class of insecticides spanning no less than 150 different compounds; are greatly in use for crops, livestock and in human health for quite some time (Tomlin, 2003; Ware, 2000). Specifically, being essential in agriculture as well as medicine (Casida and Quistad, 2004), their role in the insect control is well established (Nauen and Bretschneider, 2002). Chemicals belonging to this group are traced to dietary contaminants (Barr, 2004), in addition to causing the usual intoxication and death (Taylor, 2001). These compounds exert acute toxic effects which are mainly due to suppression of neuronal acetylcholinestrase (AChE) – whose physiological role is to hydrolyse acetylcholine, a major neurotransmitter in the peripheral and central nervous system – leading to toxicity (Sachannaa, 2003). The exact mechanism of the toxic effects of DDVP is not known. Broadly speaking, a system of enzymes called microsomal mixed-function oxidases (MFO) converts the OP insecticides containing the P=S bond

(known as thion) to P = O (known as oxon) in which the enzyme cytochrome P– 450 plays a major role (Perry, 1998). The oxons are highly toxic compounds, which account for the profound toxic effects of OP insecticides (Vijayaraghavan, 1994). The compounds show alkylating properties; and DDVP is no exception (Booth, 2007, Garret, 1990). The methyl esters have a higher alkylating potential than the ethyl esters (Garret, 1990). Alkylating agents are known to cause DNA damage (Ferguson, 1995). Most cytotoxic and genotoxic carcinogens are electrophillic by themselves or activated to electrophillic intermediates that bind to critical macromolecules (Busby and Wogan, 1984). The mutagenic activity of DDVP has not been confirmed, but may be due to the existence of electrophillic sites in the parent molecule or its metabolic intermediates are probably capable of binding to nucleophillic sites in DNA. In the DDVP, there are two potential electrophillic sites; the alkyl group/s and the phosphoryl group (Kobayashi, 2007). The OP compounds are reported to have the ability to bind to DNA (Wauchope, 1992) and cause mutations (Valkova, 1993).

On account of limited selectivity between insects and non – target species, including human displayed by OP chemicals, points to their potential adverse effects. Various OPs like Dichlorophos / Dichlorvos, Chloropyrifos, Malathion, Dimethoate, Propetamphos have caused systemic illness by AChE inhibition in humans (Sungur and Guven, 2001). Dichlorvos - 2, 2-dichlorovinyl dimethyl phosphate - is a synthetic OP insecticide. However, based on initial experimental carcinogenicity data, International Agency for Research on Cancer (IARC) has classified Dichlorvos as possible carcinogen to humans – of the level Group 2B (Ishmael et al., 2006; IARC,1991). A direct human exposure to dichorvos is expected with the use of trichlorfon, a drug employed in Alzheimer's disease treatment (Ringman and Cummings, 1999). This scenario, has forced another agency – the Environmental Protection Agency – EPA, to classify DDVP as having toxicity of Class I, meaning thereby as highly toxic and may cause cancer and tumors in rats and mice (EPA, 1991a and 1991b). These reports are serious enough to warrant a through genotoxic studies.

Hence, it is necessary to continue and extend evaluation of such chemicals in terms of genotoxicity by relevant assays sensitive in recognizing DNA damaging agents. The work on DDVP or Dichlorophos has been proposed to answer

some of the questions which need clarification. The present study is undertaken to suggest a possible mechanism of Dichlorophos *in*-vivo as genotoxic response.

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# REVIEW OF LITARATURE

Pesticides are substances used to prevent, destroy, repel or mitigate any pest ranging from insect, animals and weeds to microorganisms (Donaldson et al., 2002) have increasing utilization in the agricultural production that resulted in large amount of pesticide chemicals being released regularly into the environment and the problem of the potential genetic hazards arises due to them (Kaya et al., 2000). Many a deaths from pesticide poisoning are on record. Other deleterious effects due to pesticide exposure include reduced survival, growth and reproduction due to sub-lethal dosages and habitat reduction through elimination of various food resources (Vohora and Agarwal, 1999). The genotoxic profiles of various pesticides belonging to different chemical classes are shown in table 1. This would help to appreciate the seriousness of the problem.

Table I. Genotoxicity of pesticides: summary of the results of experimental studies

| **Chemical class ( No .of chemicals tested )** | | **Genetic endpoint** | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Gene mutation** | | | **Chromosomal mutation** | | | **DNA Damage** | | |
| | | No. of Tests | + | - | No. of Tests | + | - | No. of Tests | + | - |
| **Organophosphorous** | **24** | **23** | **15** | **8** | **22** | **20** | **2** | **22** | **17** | **5** |
| Organochlorines | 21 | 21 | 11 | 10 | 21 | 16 | 5 | 17 | 11 | 6 |
| Carbamates | 6 | 4 | 2 | 2 | 5 | 5 | 0 | 4 | 3 | 1 |
| Thiocarbamates | 10 | 8 | 6 | 2 | 7 | 6 | 1 | 9 | 6 | 3 |
| Nitroderivates | 7 | 7 | 6 | 1 | 5 | 4 | 1 | 5 | 0 | 5 |
| Pyrethroides | 6 | 6 | 2 | 4 | 5 | 5 | 0 | 3 | 2 | 1 |
| Phenoxy | 5 | 5 | 4 | 1 | 4 | 3 | 1 | 3 | 3 | 0 |
| Organoarsenic comp. | 3 | 3 | 0 | 3 | / | / | / | 1 | 1 | 0 |
| Amides | 4 | 4 | 1 | 3 | 4 | 3 | 1 | 4 | 2 | 2 |
| Bipyridil | 2 | 2 | 1 | 1 | 2 | 1 | 1 | 2 | 1 | 1 |
| Urea | 2 | 2 | 1 | 1 | 1 | 1 | 0 | 1 | 1 | 0 |
| Benzimidazoles | 2 | 2 | 2 | 0 | 2 | 2 | 0 | 2 | 2 | 0 |
| Phthalates | 3 | 3 | 3 | 0 | 3 | 3 | 0 | 3 | 3 | 0 |
| Miscellaneous | 5 | 4 | 2 | 2 | 4 | 2 | 2 | 3 | 2 | 1 |
| **% Positive results** | | | **59.6** | | | **83.5** | | | **71** | |

A cursory analysis of the table readily shows that 59.6% of the chemicals tested are active in gene mutation, while a whopping 83% inflict chromosomal damage and 71% in DNA damage; the remaining, only 10% chemicals have negative results in all tests (Bolognesi and Morasso, 2000). Discriminating among the pesticides belonging to organophosphorous insecticides (OPIs) are the most widely used synthetic chemicals in control of agricultural and domestic insects. Severity due to the organophosphorous compounds, the most widely represented chemical class - if its percentage of positive results considering the same genetic

endpoints, range from 10 to 90%, are summarized in table 2, is also a great cause of concern (Bolognesi and Morasso, 2000).

Table 2. Genotoxicity of pesticides – summary of results of experimental studies, in organophosphorous compounds.

| Organophosphorous compounds | Genetic endpoint | | | | | |
|---|---|---|---|---|---|---|
| | Gene mutation | | Choromosomal mutation | | DNA Damage | |
| | No. of tests | Positive | No. of tests | Positive | No. of tests | Positive |
| Acephate | 30 | 17 | 22 | 13 | 16 | 10 |
| Aspon[a] | **2** | **0** | / | / | / | / |
| Azynphos Methyl | 22 | 8 | 17 | 5 | 16 | 2 |
| Chlorpyrifos | 13 | 2 | 19 | 11 | 18 | 10 |
| Crotoxyphos[a] | **2** | **0** | **2** | **0** | **1** | **0** |
| Dematon | 5 | 4 | 6 | 5 | 4 | 3 |
| Diazinon | 16 | 2 | 15 | 4 | 17 | 0 |
| **Dichlorvos** | **44** | **31** | **34** | **16** | **15** | **8** |
| Dimethoate | 2 | 2 | 5 | 5 | 1 | 1 |
| Disulfoton | 8 | 2 | 3 | 0 | 5 | 2 |
| Ethion | 5 | 0 | 2 | 1 | 1 | 0 |
| Fensulfothion | 2 | 2 | 5 | 3 | 3 | 0 |
| Fenthion | 7 | 1 | 5 | 2 | 13 | 6 |
| Fonofos[a] | **4** | **0** | / | / | / | / |
| Glyphosate | 14 | 5 | 7 | 3 | 11 | 8 |
| Malathion | 25 | 4 | 34 | 24 | 20 | 12 |
| Methidathion | / | / | 3 | 2 | 2 | 0 |
| Methyl parathion | 28 | 15 | 33 | 14 | 16 | 11 |
| Monochrotophos | 23 | 12 | 25 | 20 | 13 | 9 |
| Parathion | 7 | 2 | 8 | 0 | 10 | 3 |
| Phorate | 9 | 0 | 8 | 5 | 4 | 1 |
| Phosphamidon | 9 | 5 | 7 | 7 | / | / |
| Tamaron | 1 | 0 | 4 | 0 | 3 | 2 |
| Trichlorfon | 28 | 20 | 36 | 22 | 16 | 12 |

[a] Chemical compounds negative in all tests

The use of this class of chemicals has resulted in the dreaded worldwide increases in food and fibre production, including their use in the control of major disease carrying vectors and structure damaging insect pest (Rahman et al., 2002; Chen et al., 1999). However, in addition to their intended effects, they are sometimes found to affect non-target organisms, including humans (Karabay and Og˘uz, 2005; Chaudhuri et al., 1999). Bradberry et al., (2005) have implicated this

class with widespread use in public health and agricultural programs, and thus its potential to environmental pollution, health hazards, and human poisoning is a matter of speculation. OPs also constitute a major group of chemical warfare agents, which continue to pose a global threat (Munro et al., 1994). The primary mechanism of OP toxicity is the suppression of neuronal acetylcholine esterase (AChE) in the central and peripheral nervous system, leading to a variety of short term and chronic effects (Gupta, 2006; Sachanaa, 2003). Curl et al. (2003) and Aprea et al. (2000) have together estimated that humans are universally exposed to OPs or their metabolites through environmental, occupational, and dietetic sources. The enzyme profile has also utilized to confirm the genotoxicity of organophosphorus with definite pathology in the livers of treated rats (Vijayaraghavan and Nagarajan, 1994). Organophosphorus pesticides induced apoptosis and necrosis in cultured human peripheral blood lymphocytes in *in vitro* condition are well documented (Das et al., 2006). The toxic effect at the genetic end point hold true particularly for OPIs, some of the chemicals belonging to this group are known to react with DNA and induce mutations (Moriya et al., 1983). The extensive availability of OP and potential for accidental and intentional human exposure is expected (El-Behissy, 2001).

Dichlorophos, commonly known as Dichlorvos (DDVP: 2,2-dichlorovinyl-0,0-dimethylphosphate), an OPI, has been in use for more than 40 years. Exposure to dichlorvos may occur via air, water, or food; dichlorvos is readily absorbed through all routes of exposure (Raheja and Gill, 2002). It has been evaluated in a wide range of toxicology assays including bioassays for carcinogenicity and mutagenicity (genotoxicity) and thus, has generated considerable concern due to disparity in its genotoxicity *in vitro* and *in vivo.* Its ability to alkylate DNA in bacterial, fungal or cultured mammalian cells and the consistently positive results obtained with different genetic end points show that this compound has a wide spectrum of genotoxicity *in vitro* (Booth, 2007; IPCS, 1989; NTP, 1989; Ramel *et al.,* 1980).

As far the cytotoxicity evaluations are concerned, a wide range of test systems and endpoints, including assays both *in vivo* and *in vitro* are in use. The toxic nuclear effects of Dichlorvos has shown to inflict chromosomal damage - as evidenced by increase in micronucleus and cell death - shown by decreased mitotic and replication indexes (Eroglu, 2009). They may act as genotoxic agents and may

affect various biochemical pathways (Eroglu, 2009). Studies performed on bone marrow cells in rats with various doses of dichlorvos further confirmed the mutagenic effect based on numerical and structural aberrations of chromosomes (Luty et al., 1998). The genotoxic risk of dichlorvos, in induction of tumors in mice (Benford et al., 1994), damage in bone marrow cell chromosomes in rats are reported (Yamano, 1996). The feasibility of histopathological parameter in dermally absorbed dichlorvos is also cited (Luty et al., 1998). Other study in line is made by Oral et al., (2006). Patel et al., (2007) showed the *in-vitro* cytotoxicity as well as DNA damaging potential of dichlorvos in Chinese hamster ovary cells. The *in vivo* mutagenic activity has been confirmed in the liver of transgenic mice (Pletsa, 1999). Induction of chromosomal aberrations and MN *in vivo* has been reported in Syrian hamster and rat but other work did not observe MN induction after dermal application in mouse (Mennear, 1998). DDVP has been examined in 11 long-term carcinogenicity studies in rats and mice but found to be non-carcinogenic, with the exception of the NTP studies where administered doses were very high (NTP, 1989).

The gene mutation ability of DDVP is well known (Pletsa et al., 1999). The most widely studied effects of DDVP for cytogenetic end points *in vivo* has been on the bone marrow. The effects of Dichlorophos in certain mouse strain with single or repeated exposure over a long period, indicated no significant increase in the incidence of chromosomal aberrations in bone marrow preparations inspite of using maximum tolerated dose (Dean and Thorpe, 1972). Both Kurinnyi (1975) and Moutsche-Dahmen et al. (1981) later reported a lack of clastogenic activity in the mouse after examining for CAs. The result with oral gavage dosing of DDVP for CAs in the bone marrow failed to show any increase in structural chromosome aberrations (Nehez et al., 1994). Other reports are also in line which confirms the lack of MN induction potential (Schop et al., 1990) and lack of CA (Putman and Shadly, 1992; Paik and Lee 1977) in DDVP exposed mice bone marrow cells.

Lack of DNA alkylation evidence is reported by Pletsa et al., (1999) in a range of tissues following a single intraperitoneal injection of DDVP. Methoxyphosphinyl subunit containing OPIs including Dichlorvos were assayed and a positive CA response is reported for Dichlorvos with maximum CA induction potential (Wang et al., 2003). Aberrations like chromatid gaps, sub-chromatid gaps, centromeric gaps, precocious separation of chromatids and polyploidy are

significantly higher in fish kidney cells (Rishi and Sunita, 1995). A large number of studies on cytogenetic effects on human populations exposed to pesticides are on record. Three major types of cytogenetic changes have been measured as biological indicators of genotoxic damage: chromosomal aberrations, micronuclei frequency and sister chromatid exchange. The majority of these studies gave positive results (table3) mainly as chromosomal damage (Au et al., 1999; Venegas et al., 1998).

Table 3. Genotoxicity of pesticides, human biomonitoring studies

| Exposed population | Cytogenetic endpoint | | |
|---|---|---|---|
| | Chomosomal abberation | Sister Chromatid exchange | Micronuclei frequency |
| | Number of studies +/- | | |
| Poisoned subjects | 2/0 | 2/0 | / |
| Chemical plant works | 5/1 | / | / |
| Pesticide sprayers | 8/2 | 3/4 | 0/2 |
| Agricultural workers | 5/2 | 0/4 | 0/1 |
| Floriculturists | 4/2 | 2/2 | 2/2 |
| Cotton agriculturists | 3/0 | 2/0 | |
| Vinegard workers | 2/0 | 1/0 | 1/0 |
| Total | 29/7 | 10/10 | 3/5 |
| **% +** | **90%** | **50%** | **37%** |

Based on experimental carcinogenicity data, IARC classified dichlorvos as a probable human carcinogen – Group 2B. The significant increases of forestomach tumors in mice and leukemias and pancreatic acinar adenomas in rats made the basis by the IARC and the NTP (IARC, 1991; NTP, 1989). Ishmael et al., (2006) recently reviewed the 11 carcinogenicity studies on DDVP and concluded that there is strong evidence that DDVP does not have carcinogenic activity. The investigations of Benford et al., (1994) are the most directly relevant, providing data from the forestomach of the B6C3F1 mouse that showed no evidence of genotoxicity but clear evidence of hyperplasia and cell division were not discounted. Studies by Chan et al., (1991) in Dichlorvos administered rodents reflect its carcinogenic potential associated with neoplastic responses on hematopoietic system, and various other tissues, while also a positive response for DNA damage in tissues - hematopoietic and others are on record (Sasaki et al., 2000). DNA strand breakage caused by Dichlorvos is correlated with its DNA alkylating property in cultured Chinese hamster cells (Green et al., 1974). It is also found to induce DNA damage in human lymphocytes (Atherton et al., 2006).

OPI induced biochemical and histopathological changes in different tissues is well reported by many investigators (Sutco et al., 2006; Yavuz et al., 2005; Oncu et al., 2002;). Various organs and systems affected specifically by dichlorvos are the respiratory system (Atis, et al., 2002), reproductive system (Oral et al., 2006; Okamura et al., 2005), and liver (Ogutcu et al., 2008). Histopathological and ultrastructural changes in liver, kidney, heart muscles by the dermally absorbed Dichlorvos in rats is also known (Luty et al., 1998). Acute intraperitoneal administration of sublethal doses of Dichlorvos promotes histopathological changes in kidney cells, alter renal tubular function and renal clearance of enzymes and various ions, indicating the development of acute renal disturbances (Desai and Desai, 2008). Organophosphate - induced nephrotoxicity has been established by many investigators (Bloch-Shilderman and Levy, 2007; Ben et al., 1997; Pal and Kushwah, 1997).

Human exposure to dichlorvos is inevitable due to the drug employed in the treatment of Alzheimer's disease (Ringman and Cummings, 1999). The drug spontaneously metabolizes to dichlorvos (IARC, 1991). Neurotoxicity being one of the major toxicity of organophosphorus pesticides is caused by the inhibition of acetylcholinestrase (Bajgar, 2004; Pope, 1999). It has been reported that OPs affect immune response including effects on neutrophil function (Hermanowicz et al., 1984), macrophage (Crittenden, 1998), antibody production (Johnson et al., 2002), serum complement (Casale et al., 1989), and T cell proliferation induced by IL-2 (Casale et al., 1993), concanavalin A and phytohemagglutinin both in animals and humans(Blakley et al., 1999). Depression of plasma cholinesterase is the most sensitive indicator of exposure to dichlorvos. Recent genotoxicity testing of some organophosphate insecticides, including dichlorvos by Cakir and Sarikaya (2005) showed a positive correlation with mutations. Several epidemiological studies have linked long-term exposure of OP insecticides to the development of different cancers such as non-Hodgkin's lymphoma (De Roos et al., 2003) and different leukaemias (Brown et al., 1990). OPIs are not only responsible for death due to intentional poisonings but equally contribute to high toxicity (Bhatnagar, 2001). The OP induced DNA damage at a concentration considered to be relevant for human environmental exposure is recently confirmed by Hrelijac et al., (2008). The current usage of pesticides is predicted to have effects and risks on human as well as environmental health

Conclusion drawn from all this discussion is that the current usage of DDVP can have potential effects and risks on human and environmental health. Further toxicity studies that measure other types of DNA damage, both in vitro and in vivo, are still necessary even after initial reporting (Eroglu, 2009). Several regulatory review of DDVP also stressed that there is no concern for *in-vivo* mutagenic activity. The Committee on Mutagenicity's review of DDVP concluded that there is insufficient evidence to rule out a genotoxic mechanism. Also there exist very scarce report correlating the toxicity of kidney hematopoietic cells and the bone marrow in Dichlorophos stressed *Mus musculus*. Thus, data concerning dichlorvos *in- vivo* genotoxicity is still inconclusive and their ability to induce gene mutations or chromosomal aberrations in genotoxicity assays is not very clear.

Hence, the more specific goals of the present work include to assess the *in-vivo* genotoxic potential of Dichlorophos in bone marrow; analysis of the damage in hematopoietic cells of kidney with an intention to supplement the cytogenetic findings and to compare the degree or extent of damage in the various hematopoietic organs. The study is expected in identifying the target cell type.

Chapter 3
p14 - 20

# MATERIALS AND METHODS

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- **Test organism** - Use and justification
- **Conditioning and Acclimatization**
- **Design of study and Distribution of animals**
- **Dichlorophos** - Facts and Concentration
- **Multiple Assays Performed**
  - **Micronucleus (MN) Assessment**
  - **Chromosomal Aberration (CA ) Assay**
  - **Mitotic Index (MI)**
  - **Histopathology (HP)**
- **Chemicals and Stock solutions**
- **Statistical Methods**

The protocol and related aspects of the experiments strictly followed the laid down norms for *in-vivo* genotoxicity testing in mammalian models (US/EPA 2005; OECD – 474, 1997). A brief detail of each event is described below. A more specific information will be given at relevant place ahead. We begin by animal model and its acclimatization, design of the study, some facts about the candidate compound, the parameters and statistical tools used.

- **Test organism** – Use and justification

Animal models are experimentally used as human surrogates in toxicologic evaluations. They are bioassays which involve the use of a species believed to exhibit a toxic response similar to that of humans under exposure conditions relevant to human exposure. The choice of a mammalian species we made on two counts: 1) its suitability as a model for the human experience, that is, it's close anatomic and physiologic resemblance to humans; and 2) the economic considerations, such as availability and cost of the animal.

For the assessment of acute, sub-chronic and chronic toxicity, rodent species are considered suitable, but for specific types of toxicity only certain mammals respond in a manner similar to humans. Toxicity assessment using mammalian model contributes tremendously in the extrapolation of effects observed in animal models to chemicals under laboratory conditions to humans.

*Mus musculus,* commonly known as house mouse, the one used in these studies has a standard karyotype of 20 pairs of acrocentric chromosomes including 19 pairs of autosomes and a pair of distinct X and Y chromosome. The mouse genome is about the same size as the human genome, and the organization of genes is strikingly similar between the two species. Findings from the nearly complete mouse genome sequencing project indicate that mice and humans share about 95 percent DNA sequence similarity. It means that any gene in humans is likely to have an identical or very similar counterpart (homologue) in the mouse genome. These nonhuman genomes provide powerful sets of data against which to compare the human genome. The animals in the present study put to acclimatization, before commencement of experiments.

## ▪ Conditioning and Acclimatization

The Swiss albino mice comprising single sex, the males only, procured from LABOAIDS, Meerut were brought to laboratory animal - care facility. Males were chosen since no difference in genotoxic results between the two sexes is reported for $LD_{50}$ (ip) in the literature and also on the basis of their availability. The healthy adult specimen of 8 – 10 weeks old, weighing 25 to 30 gm were used. In all experiments, animals were maintained under controlled conditions of 12 - hr dark and light period, temperature (22 ± 2°C) and humidity (70 - 80%). They were fed with commercially available sterilized pellet (Amrut Laboratory Animal Feed) and water *ad - libitum.* The procedure for acclimatization followed is the standard one conforming to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85 - 23, 1996) and kept under strict ethical guidelines.

## ▪ Design of study and Distribution of animals

Each parameter included a separate lot of 75 mice: discriminated into four experimental groups. The exposed group comprised of 45 mice in all; fifteen mice were used for each concentration employing 5 mice per duration for the test chemical. The control group consisted of normal or solvent control (distilled water) and a separate positive control (cyclophosphamide, CPA - dosed at 40 mg/kg bwt). The two control groups with 15 mice each, totalled 30 mice in all. At the end of a specific interval, 5 mice per duration were sacrificed by cervical dislocation and immediately dissected to obtain bone marrow for cytogenetic preparations and other hematopoietic organ for histopathological studies. The animals - normal and exposed - used in each parameter and duration have been illustrated in table 4.

Table 4. Animals used for various parameters

| Parameters: | Control | | Treated | | | Total |
|---|---|---|---|---|---|---|
| | Positive | Normal | I | II | III | |
| MNT,HP | 15 | 15 | 15 | 15 | 15 | 75 |
| CA,MI | 15 | 15 | 15 | 15 | 15 | 75 |
| **Durations (hr):** | | | | | | |
| 24 | 5 | 5 | 5 | 5 | 5 | 25 |
| 48 | 5 | 5 | 5 | 5 | 5 | 25 |
| 72 | 5 | 5 | 5 | 5 | 5 | 25 |

*MNT- Micronucleus test: CA: Chromosomal aberration: MI- Mitotic index: HP: Histopathology

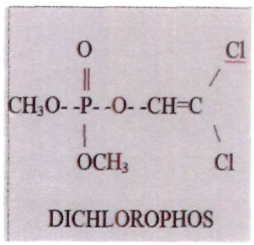
- **Dichlorophos** – Facts and Concentrations

Commercially available Dichlorvos – 76% EC DDVP Insecticide (Registration No. CIR-27,703/98/Dichlorvos/EC-698, Manufactured by Crystal Phosphates Limited) – was used in the stock solution. Dichlorvos Technical solution was based on 92% w/w. A 20µl of the stock solution was taken and raised to 1000 µl with normal saline. The stock solution was serially diluted 7 times raising the final volume to1000µl each time. The 5$^{th}$, 6$^{th}$ and 7$^{th}$ dilution was taken as third, second and first concentrations respectively for the exposure in the treatment groups. The concentrations taken in the present study on the basis of reported $LD_{50}$ for intraperitoneal mode of administration is shown in table 5. A separate fact sheet (Box I) is enclosed separately depicting its physical, chemical properties along with $LD_{50}$ reports.

Table 5. Sub-lethal Doses and Treatments

| | Dose I | Dose II | Dose III |
|---|---|---|---|
| **Sub-lethal Doses** | 10% of $LD_{50}$ | 20% of $LD_{50}$ | 30% of $LD_{50}$ |
| **Concentrations** | 0.06 mg/kg bwt | 0.08 mg/kg bwt | 0.13 mg/kg bwt |
| **Mode of administration:** Intraperitoneal (ip injection) | | | |
| **Durations:** 24, 48 and 72 hours for all the three doses | | | |

## Box I. Fact sheet: DDVP, DICHLOROPHOS*



DICHLOROPHOS

- **NOMENCLATURE**

| | | |
|---|---|---|
| COMMON NAME | : | Dichlorovos |
| IUPAC NAME | : | 2,2-Dichlorovinyl dimethyl phosphate |
| CAS NO. | : | 62-73-7 |
| CHEMICAL CLASS | : | Organophosphorous Insecticide |

- **PHYSICOCHEMICAL PROPERTIES**

| | | |
|---|---|---|
| MOL. WT. | : | 220.98 g/mol |
| MOL. FORMULA | : | $C_4H_7Cl_2O_4P$ |
| FORM | : | Colorless to amber liquid |
| MELTING POINT | : | 84°C |
| BOILING POINT | : | 140°C @ 20 mm Hg |
| VAPOURPRESSURE | : | 1.6 Pa (20°C) |
| SPECIFIC GRAVITY | : | 1.415 (25°C/4°C) |
| SOLUBITLITY(WATER) | : | 8g/L (20°C) |
| STABILITY | : | Dichlorophos is moisture sensitive. Its solutions in 95% ethanol are stable for 24 hours. Stable under normal temperature and pressure in closed container. |
| REACTIVITY | : | Incompatible with strong acids and bases, slowly hydrolyzed in acidic media and rapidly hydrolyzed by alkalis. It is corrosive to iron and mild steel. |

**APPLICATIONS**

| | | |
|---|---|---|
| MODE OF ACTION | : | Insecticide with respiratory, contact and stomach action. Gives rapid knockdown. Deplete glycogen stores due to simultaneous stimulation of glycogen phosphorylase and inhibition of glycogen synthesis. Inhibition of the esterase enzyme activity, especially cholinesterase. |
| USES | : | Control of flea (pest) collars for pets, insects in tobacco, mushroom houses, harvested tomatoes, greenhouses, animal shelters, disease vectors control, post harvest treatment of grain, household and public health fumigant, stored products and crop protection. |
| TRADE NAME | : | Dichlorvos, Vapona, Atgard, Nuvan, Task. |

- **MAMMALIAN TOXICOLOGY**

| dose | mode | species | amount | unit |
|---|---|---|---|---|
| $LD_{50}$ | ip | mus | 22 | mg/kgBwt |
| $LD_{50}$ | orl | mus | 61 | mg/kgBwt |

**TOXICITY CLASS** : IARC-2B (possible carcinogen to human)
: WHO-Ib (Highly Hazardous)
: EPA -I (Highly toxic)

*NTP CHEMICAL REPOSITORY (25 July 2001)
IARC MONOGRAHS VOLUME 53
Risk Assessment Issues for the FIFRA Science Advisory Panel (July 8, 1998)

## ▪ Multiple Assays Performed

The various parameters used in the present work are discussed. The finer detail of each protocol is discussed separately in a chapter wise fashion:

### Micronucleus (MN) Assessment

The protocol of **Schmid (1975)** was followed for the MN assessment with slight modification. Micronuclei are regarded as cytoplasmic chromatin containing bodies formed when acentric chromosome fragments or chromosome lag behind during anaphase and fail to be incorporated into daughter nuclei during cell division. Since, genetic damage result in chromosome breaks, such structurally abnormal chromosomes, or spindle abnormalities lead to micronucleus formation. The incidence of micronucleus was taken as an index of chromosome damage. Enumerations of micronuclei are much faster and technically less demanding than the scoring of chromosomal aberration.

### Chromosomal Aberration (CA) Assay

The CA test was used for the detection of structural chromosomal aberrations on exposure of the test compound. The somatic tissue used was bone marrow.

Classical protocol using bone marrow from rodents of **Preston et al., (1987)** was adopted. The extent and type of aberration was carefully studied and tabulated. Structural chromosomal aberrations of only chromosome type were documented.

### Mitotic Index (MI)

The proliferating index of the cell population, another important parameter was studied. The mitotic index was quantified as:

MI = total no. of dividing cells X 100 / total no. of cells observed

The observation excluded inter-phase – prometaphase and other substages. Inhibition of mitotic indices was taken as the cytotoxicity of the chemical. Comparison on the basis of concentration or duration of the test chemical was noted and contrasted with the controls to assess the degree of damage.

## Histopathology (HP)

The appropriate organ (kidney) of normal and insulted groups were fixed in10% neutral formalin. Tissue specimens were serially dehydrated, cleared and embedded in paraffin wax. Processed blocks were sectioned at 4 to 6μ thickness and stained by Haematoxylin and Eosin. The general histopathological observations were made. The procedure adopted, followed the method of **Bancroft et al., (1994)**.

### ▪ Chemicals and Stock solutions

**Chemicals**

- Colchicine (Sigma, Aldrich) 25 mg/50 ml of distilled water.
  ( injected at 4 mg/1000gm of body weight)
- Hypotonic solution, KCl (Merck) 0.075M; 0.56%
- Cornoys fixative (Merck) methanol : glacial acetic acid, 3:1
- May- Gruenwald stain (Merck)
- Haematoxylin (Merck)
- Eosin (Merck)
- Xylene (Merck)
- DPX (Thomas Baker)
- Foetal Bovine Serum (Sigma) 5%
- Cyclophosphamide (Merck)
  ( injected @ 40 mg/kg bwt)

**Solutions**

- Giemsa Stain (Merck)

  **Giemsa stock solution** (1gm giemsa powder added to 66 ml glycerol; incubated@ 60°C overnight; 66 ml methanol added)
  **Giemsa working solution** (1.25 ml stock solution+1.5 ml methanol + 50 ml distilled water; filtered)
- Sorensens buffer- pH 6.8(Merck)

  **Solution A** - $Na_2HPO_4.2H_2O$ 5.938 gm/100 ml
  **Solution B** - $KH_2PO_4$ 4.539 gm/100 ml
  25ml of solution A + 25ml of solution B mixed for 50 ml of solution

## ▪ Statistical Methods

All the scoring was done blindly from coded slides. The data analysis used statistical method. Mann – Whitney U Test (MWU) was performed for comparison of the relative frequencies of micronucleated erythrocytes in animals of the dose groups with those of the normal and positive control groups for MN assay. MI was expressed in percent for both the control and experimental group and $\chi^2$ test was performed to find out the significant difference between them. The frequency, percent aberrations and type of structural chromosome aberrations were recorded for each group. The mean (% ± SE) of the total aberrations was calculated, MWU test was performed for CA to determine the significant difference between mean value of the treatment groups and the concurrent controls. Statistical Package for Social Science - SPSS (16.0) software was used in the analysis of MN assessment and CA assay while MedCalc (12.0) software was used for MI data obtained in the study since comparison of proportions (MI) for $\chi^2$ analysis was lacking in SPSS.

Chapter 4
p21 - 30

# CYTOGENETIC STUDIES

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## Micronucleus Assessment

- **Potential of MNT (*in vivo*)**
- **Methodology**
- **Results**
- **Discussion**

## Micronucleus (MN) Assessment

- **Potential of MNT**

The rodent erythrocyte micronucleus assay in peripheral blood or bone marrow is the primary assay to assess *in vivo* genotoxic potential (Eastmond et al., 2009; Blakey et al., 2008; ICH, 2008). Discovered by Evans et al., (1959) – is by far easier to score MN than chromosome aberrations at metaphase. It provides a simple means for estimating induced genomic damage. Heddle (1973) and Schmid (1975) arrived independently at the same conclusion in mammalian system.

The use of micronuclei as cytogenetic end point for assessing chromosome damage is essential for three reasons. First and foremost being the scoring of damage in proliferating cells. Secondly, micronuclei persist for varying length of time after their formation so that they can be analysed in non-dividing descendents of proliferating cells. And lastly, micronuclei are produced not only as a consequence of the exclusion from daughter nuclei of acentric fragments from broken chromosomes at the time of cell division , but also from whole chromosome which may be excluded as a consequence of damage to centomere, centriole or the protein components of mitotic spindle (Evans, 1988). Not only that MN assay is considered as an alternative method to the chromosomal aberration assay by analysing metaphase chromosomes (Hayashi, 2007), but it is endowed with own merits such as:

- any dividing cell population with any karyotype can be used.
- accurate data can be obtained owing to simple end point.
- spindle poisons can also be analysed.
- background levels of micronucleated erythrocytes are low and stable.
- no additional treatment of colcemid or 5-bromo-2'-deoxyuridine, is required.

Based on the mechanism of their formation, the MN assay, in principle, is able to detect both clastogen as well as some aneugens. Hence, International regulatory agencies recognize MN assay very competent as part of a genotoxicity test battery (Krishna and Hayashi, 2000; OECD, 1997). The potential of MN induction in mammalian test system using a wide range of organophosphorous insecticides (OPI) both *in vivo* and *in vitro* studies revealed mixed results. Karabay

and Og˘uz, (2005) for example, reported the cytogenetic and genotoxic effects of the organophosphate insecticide methamidophos in the bone marrow micronucleus test in rats and showed a dose-related increase in the micronucleus frequency. The same pattern of dose-dependent increase in MN is reported by Tian and Yamauchi (2003). The genotoxic effects of four other organophosphorous pesticides by Grover and Malhi, (1985) employed the micronucleus test in bone marrow cells of the rat: where methylparathion and phorate were found mutagenic, ekatin to be weakly mutagenic and, fenitrothion produced result not different from negative control as far as MN induction potential is concerned whereas, DDVP has been found to be a MN inducer agent in human peripheral blood lymphocyte culture (Eroglu, 2009). The results of MN induction are found satisfactory in many studies (Bonassi et al., 2007; Tungul et al., 1991; Dean and Thorpe, 1972) and so considered as a good parameter of genotoxicity.

The parameter is a bridging biomarker of genotoxic exposure, capable of enumerating across multiple species including humans (Dertinger et al., 2007). The continuous advancement and expanded versatility of cytogenetic techniques as research tools, coupled with the growing imperative that the toxicity of chemicals be assessed before they can be introduced into the environment – is the basis for its use in the assessment of potential toxicity of DDVP in the present studies.

- **Methodology**

The procedural details were as per **Schmid (1975)** technique. The two control groups, normal and positive were given single intraperitoneal dose of distilled water (2 ml/100 gm bwt) and cyclophosphamide (40 mg/kg bwt). While the three DDVP exposed groups received 0.06, 0.08 and 0.13 mg/kg bwt *via* intraperitoneal injection. The dose regimen mentioned above was maintained for three time intervals, i.e. 24, 48 and 72 hr before sacrifice. Separate controls were maintained alongside. All observations were replicated thrice. The material used for slide preparation was extracted from bone marrow.

After the due processing (box I), all slides, including positive and normal controls, were independently coded and screened for analyzable micronucleus

at 40X initially, while final observation and photography was done at 100X using oil immersion. The MN appeared as deep purple in color while the cytoplasm of micronucleated cells have pink color in the normochromatic erythrocytes. In contrast, the polychromatic erythrocytes had blue to purple hue. The differential staining allowed the clear discrimination between polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs). The suitable areas were chosen for scoring where the erythrocytes were well separated, not overlapping and clearly contoured. The proportion of immature among total (immature + mature) erythrocytes by PCE/NCE ratio was determined for each replicate. 2000 immature erythrocytes per animal were scored to assess the incidence of micronucleated immature erythrocytes. Additional informations were obtained by scoring mature erythrocytes for micronuclei. The Mann-Whitney U Test (MWU) was performed using SPSS (16.0) for comparison in the relative frequencies of micronucleated erythrocytes in the dosed groups and control groups comprising normal and positive replicates. Mean (% ± SE) was further calculated for each group in these experiments.

- **Results**

The two types of erythrocytes on the basis of differential staining of May – Gruenwald – Giemsa of the bone marrow cells were observed along with the MN. The PCEs were distinct by specific purple to blue colour, while NCEs were noted with the characteristic pink shade along with PCEs. Many observations were seen with the MNPCE along with various PCEs lacking MN, clear differentiation between PCEs and NCEs were noted. The figure 1 shows these observations clearly.

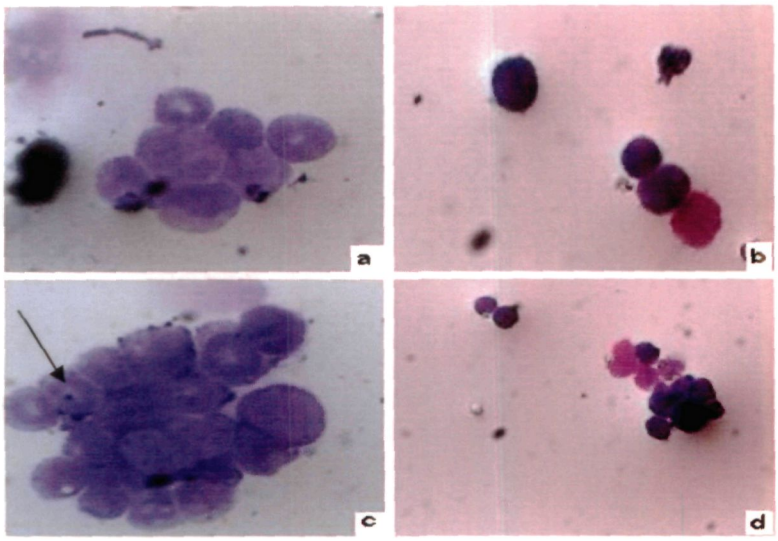


Figure 1. Bone marrow cells of mice prepared using the smear technique and stained with May – Gruenwald – Giemsa: (a), Polychromatic erythrocytes – PCEs (b), Polychromatic erythrocytes with Normochromatic erythrocytes – NCE (c), Micronucleated polychromatic erythrocyte and (d), PCEs with NCEs.

The results of micronuclei evaluation in bone marrow erythrocytes for every group per sacrifice interval are summarized in Table 6. Each data point, in the table represents the mean value for five animals per concentration of DDVP. The results demonstrate that the number of polychromatic erythrocytes containing micronuclei (MNPCEs) at each dose was not significantly increased above the concurrent normal control frequencies. No differences in the incidence of MNPCEs were observed when comparing animals treated by Dichlorophos, in increased dose concentrations with normal control, while they were significantly different from the cyclophosphamide (CPA) – the positive control. The frequency of MNPCE in the positively treated group was found to increase significantly at all time periods from the normal group. It was further observed that the animals treated intraperitoneally with aquous solution of DDVP at increasing doses, which is calculated as 10%, 20% and 30% of $LD_{50}$ (*ip*), registered no increasing frequencies of MN scored in PCE nor it depicted a time dependent increase as observed in the positively treated group. Clearly, increased numbers of micronucleated polychromatic erythrocytes was obtained using CPA, demonstrating the expected activity and sensitivity of the experimental system. The descriptive statistics of the non-parametric MWU test performed for the analysis of MN incidence in PCEs of either group is attached (Annexure 1).

Table 6. Incidences of polychromatic erythrocytes (PCEs) and Normochromatic erythrocytes (NCEs) containing micronuclei in bone marrow cells after single ip treatment of DDVP using multiple durations.

| Control groups | Time (hr) | MNPCE (% ± SE) | MNNCE (% ± SE ) | PCE/NCE |
|---|---|---|---|---|
| Normal (Distilled water) | 24 | 0.49±0.05 | 0.19±0.09 | 0.743 |
| | 48 | 0.46±0.06 | 0.23±0.05 | 0.754 |
| | 72 | 0.48±0.06 | 0.19±0.09 | 0.748 |
| | | | | |
| Positive – CPA (40mg /kg bwt) | 24 | 4.69±1.38 | 1.00±0.39 | 0.539 |
| | 48 | 5.62±1.55 | 1.58±0.87 | 0.508 |
| | 72 | 4.27±1.30 | 0.87±0.21 | 0.527 |
| **Exposed groups – Dichlorophos** | | | | |
| 0.06mg /kg bwt | 24 | 0.55±0.03 | 0.17±0.09 | 0.745 |
| | 48 | 0.43±0.07 | 0.17±0.09 | 0.750 |
| | 72 | 0.48±0.06 | 0.14±0.08 | 0.730 |
| | | | | |
| 0.08mg /kg bwt | 24 | 0.51±0.05 | 0.14±0.08 | 0.746 |
| | 48 | 0.45±0.07 | 0.21±0.09 | 0.752 |
| | 72 | 0.46±0.06 | 0.11±0.07 | 0.737 |
| | | | | |
| 0.13mg /kg bwt | 24 | 0.49±0.05 | 0.19±0.09 | 0.745 |
| | 48 | 0.45±0.07 | 0.19±0.09 | 0.721 |
| | 72 | 0.46±0.06 | 0.11±0.07 | 0.730 |

*The values are significant at 0.05 (MWU test)

The ratio of PCE/NCE or simply P/N was not affected in Dichlorophos treated animals; however, the slight depression at 48 and 72 hrs and increase at 24 hr interval reflect the normal variability rather than bone marrow toxicity. However, corresponding the P/N observations was found to be significantly decreased in the positively treated group at 24 hr and 48 hr of treatment periods showing increase of cells with MN, in contrast with the normal controls. The frequency of MNNCE for all duration and doses of DDVP treated animals was also found to be very close to the MNNCEs frequency scored in the normal group. The tabular values of mean MNPCE

(% ± SE) has been graphically represented in figure 2. It also reflects that the mean MNPCE values were significantly higher at all time intervals in the positive group while the percent frequency of micronucleated PCEs in Dichlorophos treated animals not reflected any significant increase at the specified doses or time intervals.

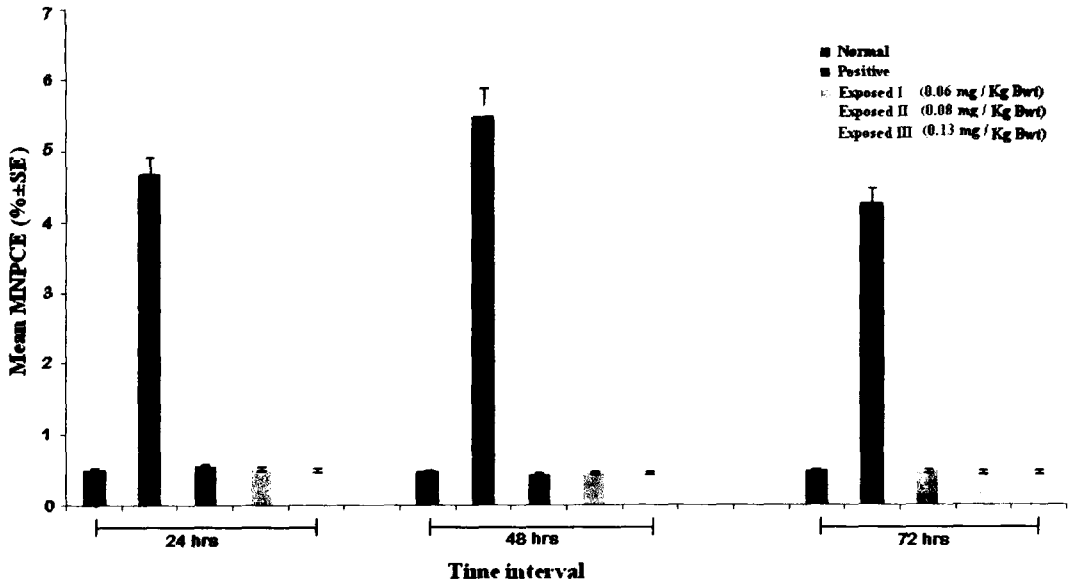


Figure 2. Mean MNPCE (% ± SE) at three different time intervals observed in the Micronucleus assay of mice bone marrow

## ▪ Discussion

The tested concentrations of Dichlorophos failed to show any significant MN induction at any dose or duration of treatment. Thus no clastogenic or aneugenic effect is expected in the treatment regimen in the bone marrow cells of *Mus musculus*. The studies done previously in the same compound also endorse the negative results through an elaborate *in vivo* mammalian MN assay (Ramel et al., 1980; Paik and Lee, 1977). The findings are further in accord with the cytogenetic investigations on mice by ip – administration of dichlorophos for 0.015 and 10 mg/kg bwt doses or doses administered by inhalation as a single exposure or even the repeated exposures. No evidence of chromosome damage or abnormalities in bone marrow cells or spermatocytes was reported either (Degraeve et al., 1984; Moutschen-Dahmen et al., 1981; Dean and Thorpe, 1972). The sole study by Dzwonkowska and Hubner (1986), demonstrated a significant increase in numbers of

cells with aberrant chromosomes in Syrian hamsters. However, these abnormalities were observed at near-lethal doses.

Since, the PCEs remain alive within the bone marrow between 10 - 33 hr (Salamone and Heddle, 1983; Cole et al., 1979), the number of MNPCEs is increased at 6 hr for aneugens and 10 hr for clastogens (Vanderkerken et al., 1989; Cole et al., 1981). Therefore, spindle poisons and clastogenic chemicals could be detected in bone marrow 24 and 48 hr after the treatment (Vanparys et al., 1992). The two periods of 24 hr and 48 hr chosen in the present design is well justified in the light of above studies which allowed sufficient window period to detect clastogens and aneugens. During this period, DDVP could not increase the number of micronucleated PCEs in any form of treatment or durations, in the mice for intraperitoneal administration. Further, no decrease in the P/N ratio was seen either. This reflecting the negative genotoxic potential in the bone marrow - the principal hematopoietic organ in mammals. DDVP treated Chinese hamsters by oral mode of administration also reciprocates our study (Dean and Thorpe, 1972). The non-genotoxic potential of Dichlorophos - *in vivo* is confirmed.

The P/N ratio is taken as a standard indication of cytotoxic effect or as an alteration in erythropoiesis. This ratio is decreased because of the cavity formation in bone marrow when there are cytotoxic effects on the cell division or due to maturation of the nucleated cells (Gollapudi et al., 1984). Another reason can be when newly maturated NCEs remain behind the bone marrow due to the failure of release into the peripheral blood on schedule (Von Ledebur and Schmid, 1973). The tested concentrations of Dichlorophos, as in our study have not significantly reduced the P/N ratio. But a significant decrease in the P/N ratio is observed in the cyclophosphamide (CPA) treated positive group. As an antitumor agent, cyclophosphamide is also known genotoxic agent in bone marrow of mice and rats (Witt et al., 2008). It is because the reactive metabolites of CPA produce DNA lesions resulting in chromosomal breakage and formation of MN (Anderson et al., 1995). The present negative results reinstate the earlier findings that Dichlorophos can be accepted as a non toxic agent under conditions relevant to human exposures.

Several factors can modulate the *in vivo* expression of Dichlorvos (Ashby, 1983). Its rapid metabolic conversion by esterase catalysed hydrolysis presumably contributes to the negative response in short term studies conducted in intact organisms (NTP, 1989; Bremmer et al., 1988). Due to the common occurrence of esterase enzymes in mammalian cells and in the blood, the hydrolytic pathway predominates over demethylation, the latter considered responsible for the genotoxic activity of dichlorvos *in vitro* (Bremmer et al., 1988).

Although the *in vivo* rodent MN assay is an effective measure of genotoxicity, it is not without limitations. The assay can only be conducted in rapidly dividing cells and typically measures chromosomal damage induced in a single tissue i.e. bone marrow. This put a limitation on assessment of genotoxic potential of a chemical (Recio et al., 2010). Since, direct measurements of chromosomal aberrations in most tissues other than blood or bone marrow are not technologically feasible, a number of surrogate endpoints are recommended to assess mutagenicity and genotoxicity in the other tissues.

## Box II. Procedure for Micronucleus Assessment
(Schmid, 1975)

MUTAGENIZED / NORMAL SPECIMENS

⇩

FEMUR DISECTED

⇩

BONE MARROW CELLS (BMCs) IN NORMAL SALINE

(30 min; 37°C)

⇩

BONE MARROW CELLS (BMC) IN FCS/FBS

⇩

CENTRIFUGATION

(5 min; 1000 rpm)

⇩

PELLET IN FCS

(save)

⇩

CENTRIFUGATION

(5 min; 1000 rpm)

⇩

SMEAR

(Grease free slide)

⇩

AIR DRIED

(Overnight)

⇩

FIXATION

(5 min; Methanol – 100%)

⇩

STAINING in May – Gruenwald / Giemsa

(25 min)

⇩

OBSERVATION

(40X; 100X)

**SPSS 16.0 version Output sheet**

NPar TESTS

MWU= MN BY VAR00002 (1 2)

Descriptive Statistics

ANNEXURE 1

[DataSet0]

| | N | Mean | Std. Deviation |
|---|---|---|---|
| MN | 15 | 79.6000 | 122.31096 |
| VAR00002 | 15 | 1.8000 | .41404 |

Mann-Whitney Test

| Ranks | | | | |
|---|---|---|---|---|
| | VAR00002 | N | Mean Rank | Sum of Ranks |
| MN | 1 | 3 | 7.67 | 23.00 |
| | 2 | 12 | 8.08 | 97.00 |
| | Total | 15 | | |

| Test Statistics[b] | |
|---|---|
| Mann-Whitney U | 17.000 |
| Wilcoxon W | 23.000 |
| Z | -.146 |
| Asymp. Sig. (2-tailed) | .884 |
| Exact Sig. [2*(1-tailed Sig.)] | .945[a] |

a. Not corrected for ties.

b. Grouping Variable: VAR00002

Chapter 4
p31 - 42

# CYTOGENETIC STUDIES

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## Chromosomal Aberration (CA) Assay and Mitotic Index (MI)

- **Relevance of CA and MI**
- **Methodology**
- **Results**
- **Discussion**

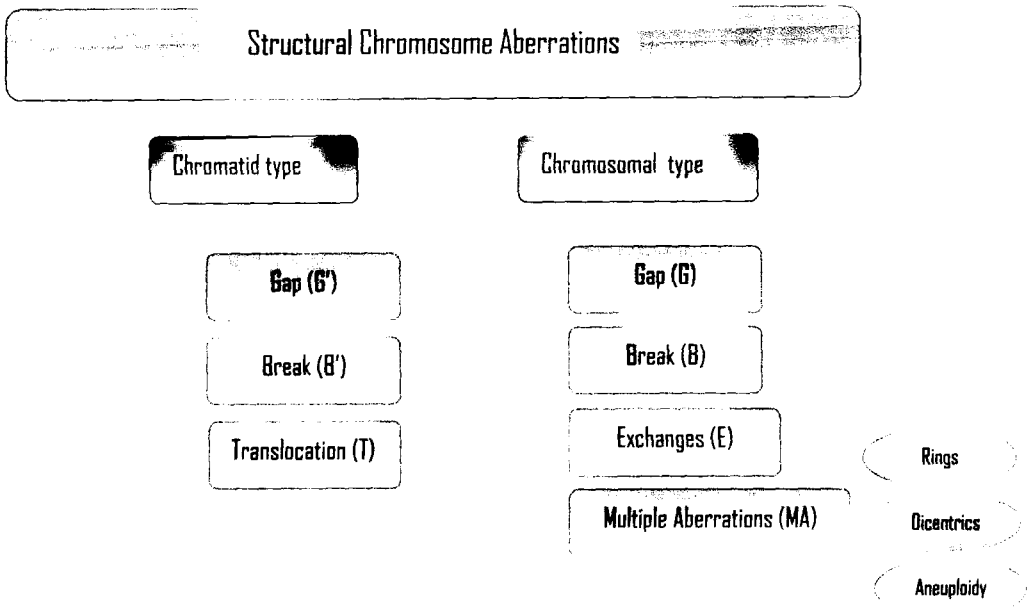
## Chromosomal aberration (CA) Assay and Mitotic Index (MI)

- **Relevance of CA and MI**

The chromosome aberration (CA) assay has been effectively used to find structural chromosome aberrations induced by chemical compounds in bone marrow cells of rodents (Tice et al., 1994; Richold et al., 1990; Preston et al., 1987; Adler, 1984). It is quite sensitive and useful to assess the potential genotoxicity of many compounds (Yilmaz et al., 2008), and considered as one of the markers of early biological effects of carcinogens (Liou et al., 2002).

The organophosphate induced clastogenic studies are many (Karabay and Og˘uz, 2005; Jayashree et al., 1994). Other OP compounds, including phosphamidon, investigated by *in vivo* CA in mice using multiple modes of administrations, tested positive for genotoxicity (Behera and Bhunya, 1987). Yet again, CA in five other organophosphorus insecticides including Dichlorvos gave similar results in Chinese hamster ovary cells Wang et al., (2003).

Classification of chromosomal aberrations (Adler, 1980) was used in present studies. The structural type of aberrations studied under two further sub – types, chromatid and chromosome. The former type involved one chromatid, showing chromatid gap (G′), achromatic lesion with a size smaller than the width of one chromatid; chromatid breaks (B′) having the true discontinuities with clear dislocated fragment and translocations (T), where the fragments almost remained associated with chromosome of origin. The chromosome aberration, involved both chromatids at identical sites distinguishable in gap (G), an achromatic lesion, having non – staining region not greater than the diameter of the chromatid; break or (terminal deletion) that involved only one chromosome; chromosomal exchanges, where two or more lesions in the same or different chromosomes were observed. The remaining others classified as multiple aberrations (MA) comprised of rings, dicentrics and aneuploidy, where the chromosome number of a cell deviated from a haploid set, 20 in the present case. Remaining abnormalities, improper spreading, clumping or stickiness (S) and pulverization (P), were scored separately. The broad outline of classification followed was:




Mitotic index (MI) is another test, indicative of cytotoxicity of chemicals in CA assay. A decreased MI is taken as the inhibition of cell cycle and affecting the cell division negatively (Amorim et al., 2000), an increase in MI is indicative of accumulation of mitoses because of disturbance of spindle function (Sbrana et al., 1993). The superlative effect of aberration analysis and micronucleus assay allows to distinguish between a positive MN response due to chromosomal breakage and that obtained by lagging chromosomes due to spindle impairment (Wang et al., 1990).

- **Methodology**

The standard protocol of **Preston et al., (1987)** was followed as shown in flowchart (Box II). After the completion of a definite exposure to the concentrations, the animals were injected intraperitoneally with the colchicine at the rate of 4 mg/kg bwt, two hrs prior to sacrifice. The bone marrow cells were collected from both the femurs by flushing in warm (37°C) 5ml KCl (0.56%) in a centrifuge tube. Following proper homogenization, the tissue was incubated at 37°C in a water bath for 18 min. Incubation of the material was followed by centrifugation at 1000 rpm for 10 min. The supernatant was decanted and the cell pellet was saved. Thereafter, it was fixed with cold aceto – methanol fixative (glacial acetic acid / methanol, 1:3 v/v).

Centrifugation and fixation was repeated twice with an interval of 30 min. For slide preparations, two to three drops of the material (centrifuged and re – suspended in 2 ml of the fixative) were dropped from appropriate height on a clean, grease – free slide previously chilled in 50% ethanol; the slides ignited on a smokeless flame (spirit lamp) to burn off the fixative, air – dried and stored in a dust – free condition until staining.

The staining was preferred on the following day in 5% buffered (Sorenson's; pH – 6.8) Giemsa (pH – 7.0) for 10 min and again air – dried and mounted in DPX and randomized. All slides were coded and scored blindly by a single observer. The slides were screened for analyzable metaphases fulfilling the criteria; have fully aligned chromatid, no centomere splitting, no extensive overlap of chromosomes and proper fixation and staining. Well separated metaphases were analysed and observed initially under low power (10X, 45X) and subsequently at high magnification (100X). CAs was scored blind – fold and at least 100 well spread metaphases per animal were scored. The gaps were excluded from the total aberration count because it is assumed that gaps are sites of despiralization in the metaphase chromosome that render the DNA invisible under light microscopy (IPCS, 1985) and also that an achromatic lesion may actually be a single – strand break in the DNA double helix as a result of incomplete excision repair and, thus, it may represent a point of possible instability (IPCS, 1985). However, the cause of the gap formation is yet not understood fully. We, therefore, reported the gaps separately from true chromosome aberrations.

The mitotic index was determined by counting the metaphase cells from approximately 1000 cells per animal for exposed replicates. Similar observations were carried out for positive control and untreated normal controls. The mitotic index was quantified as:

MI = Total no. of dividing cells X 100 / Total no. of cells observed

The observation excluded interphase – prometaphase and substages. Inhibition of mitotic indices was taken as the cytotoxicity of the chemical. Comparison on the basis of concentration and durations of the test chemical was noted and contrasted with the controls to assess the degree of damage.

The frequency, percent aberrations and type of aberrations were recorded for each group. The mean (% ± SE) of the total aberrations was calculated. Statistical tests were performed by using SPSS (Statistical Package for Social Science) version 16.0 for MWU test and software MedCalc, version 12.0 for Chi – square test. The descriptive statistics of the non – parametric – MWU test performed for the analysis of CA incidence in the treated (including positive) and normal control group is also enclosed (Annexure 2). Output sheet of Chi – Square ($\chi^2$) for MI analysis is attached (Annexure 3).

- **Results**

Many types of aberrations were observed in Dichlorophos and CPA treated replicates. That included breaks, fragments, exchanges, and multiple aberrations like dicentrics, gaps, stickiness and pulverization. Some of important aberrations seen in Dichlorophos treated animals are shown in the figure 3.

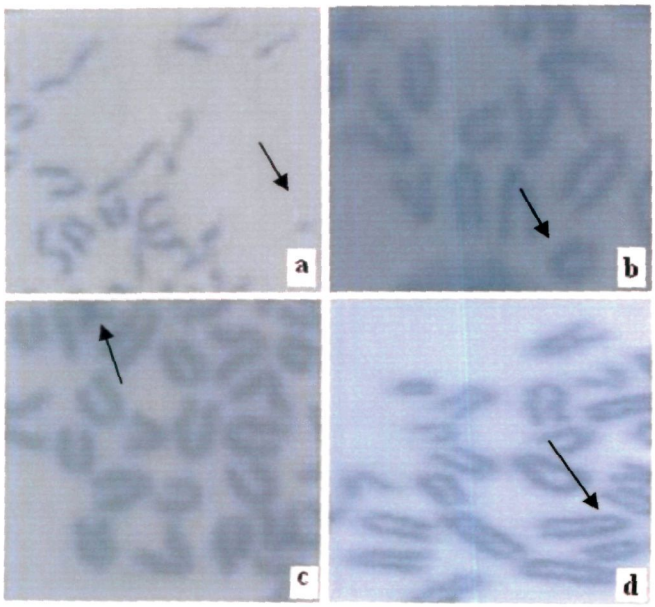



Figure 3. Some of the chromosomal aberrations (arrow) observed in bone marrow cells of Dichlorophos treated mice: Break (a); Ring chromosome (b); Exchange (c); Dicentric (d). (Magnification 100X).

Dichlorvos did not induce a significant increase of chromosomal aberrations in the mice at any concentrations and treatment periods when compared to the normal control (Table 7). Total aberration seen in the positive groups, i.e. 12.96 ± 3.01, 18.35 ± 4.08 and 17.56 ± 3.93 are much higher than corresponding values in normal, which are 1.06 ±0.53, 0.73 ± 0.07 0.47 ± 0.06 at respective intervals.

Table 7. Effect of various doses of Dichlorophos on the metaphase chromosomes of bone marrow cells of *Mus musculus* after multiple durations.

| Control Group | Time (hr) | SMC | Number and type of chromosomal aberrations | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Breaks | | Rings | | Exchanges | | Dicentrics | | S & P | | Gaps | | Total (excluding gap) | | Total (%±SE) |
| | | | no. | % | no. | % | no. | % | no. | % | no. | % | no. | % | no. | % | |
| **Normal** (Distilled Water) | 24 | 497 | 2 | 0.4 | 1 | 0.2 | 0 | 0 | 1 | 0.2 | 0 | 0 | 0 | 0 | 4 | 0.8 | 1.06±0.53 |
| | 48 | 492 | 1 | 0.2 | 0 | 0 | 0 | 0 | 1 | 0.2 | 0 | 0 | 1 | 0.2 | 2 | 0.4 | 0.73±0.07 |
| | 72 | 495 | 0 | 0 | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.2 | 0.47±0.06 |
| **Positive – CPA** (40 mg/kg bwt) | 24 | 488 | 18 | 3.68 | 9 | 1.84 | 1 | 0.2 | 3 | 0.61 | 8 | 1.63 | 1 | 0.2 | 39 | 7.99 | 12.96±3.01 |
| | 48 | 490 | 29 | 5.91 | 13 | 2.65 | 3 | 0.61 | 1 | 0.2 | 9 | 1.83 | 0 | 0 | 55 | 11.22 | 18.35±4.08 |
| | 72 | 493 | 19 | 3.85 | 22 | 4.46 | 6 | 1.22 | 0 | 0 | 6 | 1.21 | 1 | 0.2 | 53 | 10.75 | 17.56±3.93 |
| **Exposed – Dichlorophos (mg /kg bwt)** | | | | | | | | | | | | | | | | | |
| **0.06** | 24 | 487 | 3 | 0.61 | 1 | 0.2 | 1 | 0.2 | 0 | 0 | 0 | 0 | 1 | 0.2 | 5 | 1.02 | 1.11±0.92 |
| | 48 | 484 | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.2 | 0 | 0 | 2 | 0.41 | 0.73±0.08 |
| | 72 | 468 | 1 | 0.21 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.21 | 0 | 0 | 1 | 0.21 | 0.28±0.06 |
| **0.08** | 24 | 490 | 2 | 0.4 | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 0.61 | 0.93±0.28 |
| | 48 | 479 | 2 | 0.41 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0.41 | 0.73±0.08 |
| | 72 | 484 | 1 | 0.2 | 0 | 0 | 1 | 0.2 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0.41 | 0.48±0.06 |
| **0.13** | 24 | 480 | 0 | 0 | 1 | 0.2 | 0 | 0 | 1 | 0.2 | 1 | 0.2 | 0 | 0 | 3 | 0.625 | 0.95±0.31 |
| | 48 | 491 | 0 | 0 | 1 | 0.2 | 1 | 0.2 | 0 | 0 | 0 | 0 | 1 | 0.2 | 2 | 0.407 | 0.74±0.08 |
| | 72 | 482 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0.2 | 0 | 0 | 1 | 0.207 | 0.48±0.06 |

*Values are significant at 0.05 (MWU test); S = Stickiness; P = Pulverization.

The mean aberration (% ± SE) values of dichlorophos treated animals when compared to normal, is found to be almost close to that obtained for the normal control. The mean rank for normal control as per MWU test is 6.33 while for the treated animals (including positive) the value obtained is 8.42, indicating that the treated replicates scored higher than the normal control. The U statistic obtained i.e. 13, is the number of times items of the lower rank group (normal) go behind items of higher ranked group (exposed). The Z is the standardized score associated with the significance value ($p = 0.536$). Since the p value is larger than Z score, we conclude that normal control did not score significantly higher than the treated group of test animal (Annexure 2).

A graphical representation of the total aberration scored (% ± SE) has been shown in figure 4 in the normal, positive and Dichlorophos exposed animals at the usual three doses in increasing order of concentrations at multiple exposures. No variation in percent aberrations is seen when the drug treated animals are compared to the normal ones, while a marked increase in total aberration percent is clearly visible at all time periods of treatment in the positive group in contrast to the normal and DCV treated animals.

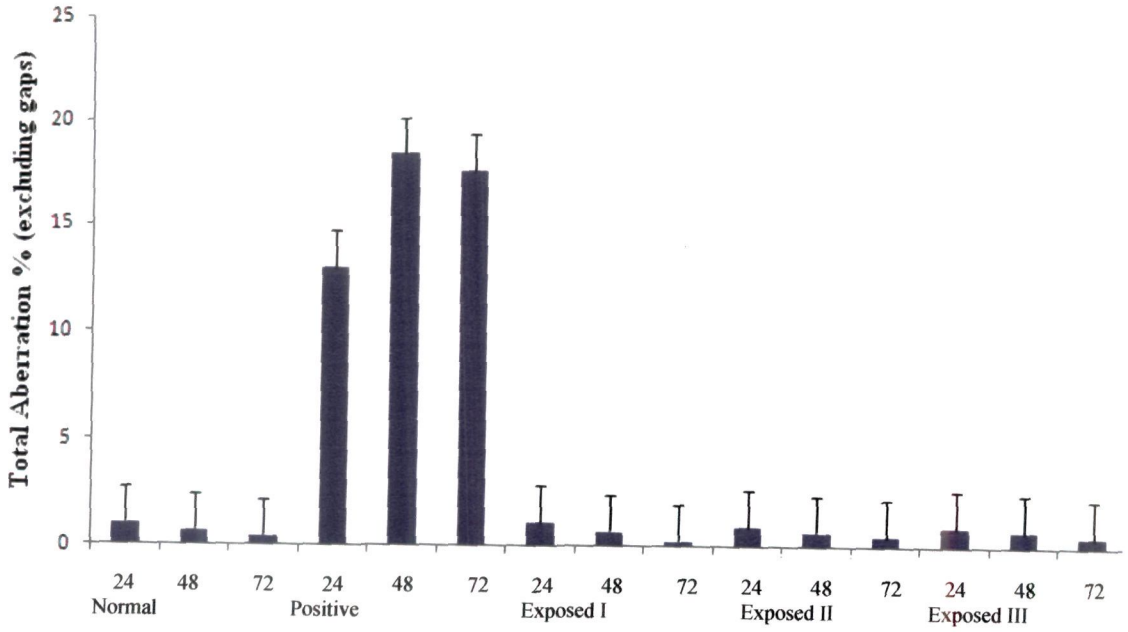



Figure 4. Total aberration (% ± SE) excluding gaps in the normal, positive and Dichlorophos exposed *Mus musculus* after single ip treatment at 24, 48 and 72 hr. Exposed I (0.06 mg/kg bwt); Exposed II (0.08 mg/kg bwt); Exposed III (0.13 mg/kg bwt).

The percent MI as observed, calculated and recorded in table 8. There was no significant difference between Dichlorvos treated groups and the normal control group in the percentages of mitotic indices of bone marrow cells at any dose levels or at any time intervals used in the present study. However, the MI% was found to be highly significant and increased in the positive controls as compared to that of the dosed and normal control. The chi – square test when performed on the aggregate MI proportions also confirmed the results obtained in the present case. A difference of 4.05% (P= 0.0423) was obtained when positive was compared to the normal group, meaning thereby, significant difference exist between them and hence, a decrease in MI (%) in positive group is supplemented statistically. Likewise, similar trend is observed when the MI aggregate proportions of positive group is compared to DDVP exposed replicates at three doses, and $\chi^2$ values of 4.04, 3.95 and 3.74 respectively (for increasing dose concentrations) is obtained. This also confirms that the decrease in MI of positive groups was significantly higher in contrast to the exposed replicates. Further, the MI result is strengthened when normal and exposed replicates are compared, where, very less difference (0.01%, 0.1% and 0.31% for three increasing dose concentrations of DDVP in exposed specimen) with corresponding $\chi^2$ values of 0.005, 0.000038 and 0.0106 are obtained. It is concluded that the MI (%) of the normal and DDVP treated animals do not differ significantly (Annexure 3).

Table 8. Mitotic index (%) in bone marrow cells of *Mus musculus*

| Control Group | Time | Total cell No. | No. of dividing cells | MI (%) |
|---|---|---|---|---|
| **Normal** (Distilled water) | 24 | 5000 | 244 | 4.88 |
| | 48 | 5000 | 248 | 4.96 |
| | 72 | 5000 | 251 | 5.02 |
| **Positive – CPA** (40mg /kg bwt) | 24 | 5000 | 126 | 2.52 |
| | 48 | 5000 | 143 | 2.86 |
| | 72 | 5000 | 158 | 3.16 |
| **Exposed group – Dichlorophos (mg/kg bwt)** | | | | |
| 0.06 | 24 | 5000 | 245 | 4.90 |
| | 48 | 5000 | 249 | 4.98 |
| | 72 | 5000 | 261 | 5.22 |
| 0.08 | 24 | 5000 | 242 | 4.84 |
| | 48 | 5000 | 251 | 5.02 |
| | 72 | 5000 | 252 | 5.04 |
| 0.13 | 24 | 5000 | 238 | 4.76 |
| | 48 | 5000 | 253 | 5.06 |
| | 72 | 5000 | 246 | 4.92 |

*Values are significant at 0.05 ($\chi^2$ test)

- **Discussion**

Clastogenic chemicals are supposed to cause abnormalities at the chromosome level and detected by the conventional *in vivo* CA assessment in the mammalian system. The tested concentrations of Dichlorvos could not significantly induce CAs in the bone marrow cells. The MI is used as the cytotoxic measure in the CA assay. The enhanced MI arise from interference with spindle apparatus or with protein synthesis, indicative of increased cell proliferative activity while decreased MI indicates lesser number of cells completing the cell cycle and hence a high proportion of cells belonging to the resting stage of the cell cycle (Verma and Purnima, 1992). In the present experimental conditions, however, Dichlorophos treated animals showed no marked decrease or increase in the MI when contrasted with normal. It is suggestive of that DDVP neither inhibits nor induce mitotic progression.

The negative CA results obtained, beside MI, is in concurrence with the earlier studies in mice bone marrow (Degreave et al., 1984; Paik and Lee, 1977; Dean and Thorpe, 1972). In a rare *in vitro* study where human lymphocytes were treated with Dichlorvos, a negative response in the CA analysis is reported while oral feeding of DCV in female *Drosophila melanogaster* reflected positive (Moutschen–Dahmen et al., 1981). However, it is not to be strictly compared given the test animals widely differed. The negative results of the present study as well as of earlier studies could be due to the fact that DCV is rapidly metabolized by esterase *in - vivo.* As the phosphate group in DCV is more electron – withdrawing than the methyl group and that is reacts to esterase more than DNA in the blood and tissues, it is *not* likely to induce genotoxicity *in vivo* ( Wright,1978)

Therefore, DCV shows *in vitro* genotoxicity without metabolic activation but seldom exhibit *in vivo* genotoxicity. The clastogenicity of the positive test chemical, CPA found in the present CA assay is in conformity to usually positive results.

## Box III. Procedure for Mammalian Bone marrow chromosomal aberration

(Preston et al., 1987)

NORMAL / TREATED

⇩

COLCHICINE TREATMENT
(ip; 2 hrs prior to blood collection)

⇩

ANIMAL SACRICED & FEMUR COLLECTED

⇩

FLUSHING BMCs* WITH PREINCUBATED
0.56% KCl

⇩

BMCs INCUBATION
(37°C; 18 min)

⇩

CENTRIFUGATION
(1000 rpm; 10 min)

⇩

PELLET
(save)

⇩

FIXATION
(Fixative 3:1)

⇩

CENTRIFUGATION
(1000 rpm; 5min; Twice)

⇩

PELLET
(save in 2ml Fixative) – (BMCs Suspension)

⇩

DROPINGS BMCs SUSPENSION
(on pre – cleaned, chilled ethanol – 50%, dipped slides)

⇩

FLAME DRY & AIR DRY
(Overnight)

⇩

STAINING
(in buffered Giemsa 10 min)

⇩

MOUNTING; CLEARING
(DPX; Xylene)

⇩

OBSERVATION
(40X; 100X with oil immersion)

*Bone marrow cells

# SPSS 16.0 version Output sheet

NPar TESTS

MWU = CA BY VAR00002 (1 2)

Descriptive Statistics

ANNEXURE 2

**NPar Tests**

[DataSet0]

| | N | Mean | Std. Deviation |
|---|---|---|---|
| CA | 15 | 11.6667 | 19.63112 |
| Grouping | 15 | 1.8000 | .41404 |

**Mann-Whitney Test**

| | Grouping | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| CA | 1 | 3 | 6.33 | 19.00 |
| | 2 | 12 | 8.42 | 101.00 |
| | Total | 15 | | |

| **Test Statistics**[b] | |
|---|---|
| | CA |
| Mann-Whitney U | 13.000 |
| Wilcoxon W | 19.000 |
| Z | -.738 |
| Asymp. Sig. (2-tailed) | .460 |
| Exact Sig. [2*(1-tailed Sig.)] | .536[a] |

a. Not corrected for ties.

b. Grouping Variable: Grouping

# SPSS 16.0 version Output sheet

NPar TESTS

MWU = CA BY VAR00002 (1 2)

Descriptive Statistics

ANNEXURE 2

**NPar Tests**

[DataSet0]

| | N | Mean | Std. Deviation |
|---|---|---|---|
| CA | 15 | 11.6667 | 19.63112 |
| Grouping | 15 | 1.8000 | .41404 |

**Mann-Whitney Test**

| | Grouping | N | Mean Rank | Sum of Ranks |
|---|---|---|---|---|
| CA | 1 | 3 | 6.33 | 19.00 |
| | 2 | 12 | 8.42 | 101.00 |
| | Total | 15 | | |

| **Test Statistics**[b] | CA |
|---|---|
| Mann-Whitney U | 13.000 |
| Wilcoxon W | 19.000 |
| Z | -.738 |
| Asymp. Sig. (2-tailed) | .460 |
| Exact Sig. [2*(1-tailed Sig.)] | .536[a] |

a. Not corrected for ties.

b. Grouping Variable: Grouping

ANNEXURE 3

Comparison of proportions of aggregate MI (%) for Chi-square ($\chi^2$) test

| Group I | Group II | Difference (%) | $\chi^2$ Value | Significance level (P) |
|---|---|---|---|---|
| Normal | Positive | 4.05 | 4.124 | 0.0423 |
| Positive | Exposed I | 4.04 | 4.104 | 0.0428 |
| Positive | Exposed II | 3.95 | 3.916 | 0.0478 |
| Positive | Exposed III | 3.74 | 3.516 | 0.0608 |
| Normal | Exposed I | 0.01 | 0.005 | 0.9428 |
| Normal | Exposed II | 0.1 | 0.00038 | 0.9844 |
| Normal | Exposed III | 0.31 | 0.0106 | 0.9180 |

Positive: 40 mg/kg bwt of CPA; Exposed I: 0.06 mg/kg bwt of DDVP; Exposed II: 0.08 mg/kg bwt of DDVP; Exposed III: 0.08 mg/kg bwt of DDVP

Chapter 5
p43 - 53

# HISTOPATHOLOGICAL ASSESSMENT

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- **Significance of histopathology**
- **Specimen preparation for light microscopy**
- **Observations and results**
- **Discussion**

## ▪ Significance of Histopathology

Evaluation of the pathological alterations induced by the chemical agent in the experimental model represents the cornerstone of their safety assessment. This preliminary assessment, based on conventional histopathological techniques, represents a major contribution to the overall genotoxic potential of the test agent in mammalian system, hence vital for extrapolation in human (Greaves, 2011). The results of conventional toxicological assays along with pathology are sufficient to predict important adverse effects *in vivo*, in terms of possible toxicity induced by a chemical. A good co-relation exists between the genotoxic results obtained by cytogenetic techniques and histopathological examination of tissues when obtained from the same experimental group (Wanda et al., 2010). Histopathology, thus, represent one of the most sensitive technique to detect any toxicity signs and symptoms or adverse effects of mammalian tissue

Given to the human exposure to Dichlorvos, its carcinogenic potential evaluations become mandatory. A study showed sufficient evidence of an association between Dichlorvos exposure and human cancer risk (Koutros et al., 2008). Very limited studies appear to have been done on its toxicity to kidney hematopoietic tissues, rather most of the studies conducted on blood and bone marrow (Eroglu, 2009). It is well known that organophosphorous insecticide and their metabolites are eliminated through urine and are likely to affect nephrons. Although organophosphate – induced nephrotoxicity is well reported by many investigators (Bloch-Shilderman and Levy, 2007; Ben et al., 1997; Mantle et al., 1997), but reports on DDVP induced toxicity in hematopoietic kidney cells are very rare. A recent report by Desai and Desai (2008) suggests that a single acute dose of DCV promotes necrosis of renal tissue, thereby altering the normal physiological functions of nephrons.

In, this light, pathological evaluation of hematopoietic cells of kidney in the genotoxic assessment of DDVP can provide insight into the potential toxicity issues. A review of the NTP data base also suggests that potent genotoxicants are capable of inducing tumors in characteristic multiple sites in rodent studies. The relevant information is scattered in pathology literature specially of rodent tumor studies which is of great significance in human. Hence, genotoxic agent – induced results supported by conventional toxicological and histopathological studies are of much prognostic value for human.

- **Specimen preparation for light microscopy**

The processing for kidney tissue and preparation of sections for light microscopic study followed the protocol of Bancroft et al., 1994. The method along with some modification in the standard protocol is summarized in box iv.

After sacrificing the Swiss albino mice, small piece of cortical kidney was extirpated and fixed in aqueous Bouin's solution. The tissue was fixed, washed with distilled water and routinely dehydrated by upgrading alcohol series from, 30%, 50%, 70% (½ hour each), and then from 90% to 100% (two changes of ½ hour each). Clearing of the tissue is carried in xylene for 10 – 15 min, followed by paraffin infiltration and embedding in two steps – transferring tissue to xylene + wax (1:1), for 15 min and then, in wax (100%) for 15 min. Block preparation of tissue with proper orientation and trimming was carried out carefully. Block was fixed on the block holder (object disc) of rotary microtome and 4 – 6μ thick sections were cut and transferred to albumin – smeared slide, followed by overnight affixing and stretching of the sections on hot plate at 45°C. Sections were thereafter dipped in xylene for 10 min and then, rehydrated by downgrading in alcohol series. After washing with distilled water, sections were stained in hematoxylin (10 min), washing with distilled water again, followed by staining in eosin for 1– 2 min. This is again cleared in xylene and finally, mounted with DPX using coverslip.

Study of the slides were done at 10X, 40X and 100x with oil immersion and photographed. Histopathological changes were identified and evaluated in the presence of an expert of pathology in the University Medical College (JNMC) and Hospital, AMU. Attention was given on glomerulus, proximal convoluted tubules (PCT – identified as eosinophillic cuboidal epithelium with centrally located nuclei) and distal convoluted tubules (DCT – identified as basophilic cuboidal epithelium with apical nuclei).

- **Observations and Results**

Changes observed in the hematopoietic kidney cells of positively treated and test chemical exposed mice are recorded (table 9). These changes are observed in the cortical kidney of mice. Representative illustrations showing normal and modified histopathological forms are shown in figures 5 – 7.

Table 9. Histopathological changes observed in the hematopoietic kidney cells of *Mus musculus*.

| Groups | Time (hr) | OBSERVATIONS* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | GL | PTA | PTN | DTA | DTN | IO | TLECD | INIF | TR |
| **Positive – CPA** (40 mg/kg bwt) | 24 | Moderate | Moderate to Severe tubular degeneration and necrosis | | | | Moderate | Highly significant amount | Moderate | Moderate |
| | 48 | Moderate to severe oedema | Severe tubular degeneration and necrosis | | | | Severe | Significant amount | Moderate | Moderate |
| | 72 | Mild oedema | Moderate to Severe tubular degeneration and necrosis | | | | Moderate to Severe | Moderate to significant | Mild | Mild to moderate |
| **Exposed Group – Dichlorophos (mg /kg bwt)** | | | | | | | | | | |
| 0.06 | 24 | Normal | Mild | Mild | Mild | Mild | Mild | Scant amount | – | Very scant |
| | 48 | Apparently normal; Mild oedema | Mild | Mild | Mild | Mild | Mild | Scant amount | Mild | Scant |
| | 72 | Normal | Normal | – | Normal | Normal | Normal | Very scant amount | Very scant | – |
| 0.08 | 24 | Mild oedema | Moderate | Moderate | Moderate | Moderate | Moderate | Moderate | Mild | Mild |
| | 48 | Mild oedema | Moderate to severe | Moderate | Moderate | Moderate | Moderate | Moderate amount | Mild | Mild to Moderate |
| | 72 | Apparently Normal | Mild to moderate | Mild to moderate | Mild | Mild | – | Very scant amount | Very scant | Scant |
| 0.13 | 24 | Moderate oedema | Severe | Severe | Severe | Severe | Severe | Significant amount | Mild | Moderate |
| | 48 | Mild oedema | Moderate to severe | Moderate to severe | Severe | Moderate to severe | Moderate to Severe | Significant amount | Mild | Mild to Moderate |
| | 72 | Mild oedema | Mild | Mild | Moderate | Mild | Mild | Moderate | Very scant | Mild |

* GL- Glomeruli; PTA- Proximal tubular atrophy; PTN- Proximal tubular necrosis; DTA- Distal tubular atrophy; DTN- Distal tubular necrosis; IO: Interstitial oedema; TLECD: Tubular luminal eosinophilic cytoplasmic debris; INIF- Interstitial inflammation: TR- Tubular regeneration.

Lack of noticeable loss or gain of kidney weight in the Dichlorophos treated (0.06, 0.08 and 0.13 mg/Kg Bwt) Swiss albino male mice was observed. The untreated control showed typical normal renal morphology (Fig. 5 a – c).

24 and 48 hr kidney sections of CPA treated positive specimen show moderate to severe glomerular oedema along with the same scale of tubular degeneration and necrosis (Fig. 6 a – b). The proximal and distal tubules cannot be made out separately due to severe degenerative and atrophic changes. While at 24 hr, kidney sections of dichlorophos concentration – 0.06 mg/kg bwt, showed normal glomeruli with mild tubular atrophy and degeneration (Fig. 7a), mild interstitial oedema, IO (Fig. 7c) and, at 48 hr – exhibited apparently normal glomeruli (Fig. 7b) to mild glomerular oedema, mild proximal and distal tubular atrophy and necrosis with very scant tubular luminal eosinophilic cytoplasmic debris (TLECD) at 48 hr (Fig. 7a, c) along with mild IO (Fig. 7c) and mild interstitial inflammation (INIF) (fig. 7d). The toxicity symptoms decreased and normal histology was observed at 72 hr for the same dose. In contrast, ip administration of higher doses – 0.08 mg/kg bwt and 0.13 mg/kg bwt, induced mild while exhibiting moderate – oedema of glomeruli at 24 hr respectively (Fig. 8a and 9a). However, at 48 hr, both the doses showed mild glomerular oedema which diminishes with increasing time and reach normal morphology at 72 hr.

Kidney sections of mice treated with dichlorophos reflects dose – dependent damages, in terms of tubular degeneration and atrophy, wherein the changes were seen to be mild at lower dose moving on to moderate at next higher dose while being severe at the highest concentration, also exhibit absence, then mild and finally moderate glomerular oedema along with increasing dichlorophos concentration. The dose – dependent response of the toxic insult induced in these hematopoietic cells was also supported by the mild interstitial oedema at lowest dose, moderate at next higher concentration while observed to be severe at the maximum selected dose. Mice exposed to highest concentration, 0.13 mg/kg bwt of Dichlorophos, for 24 and 48 hr, showed signs of severe tubular degeneration and necrosis and significant amount of tubular interstitial eosinophilic debris (Fig. 9c).

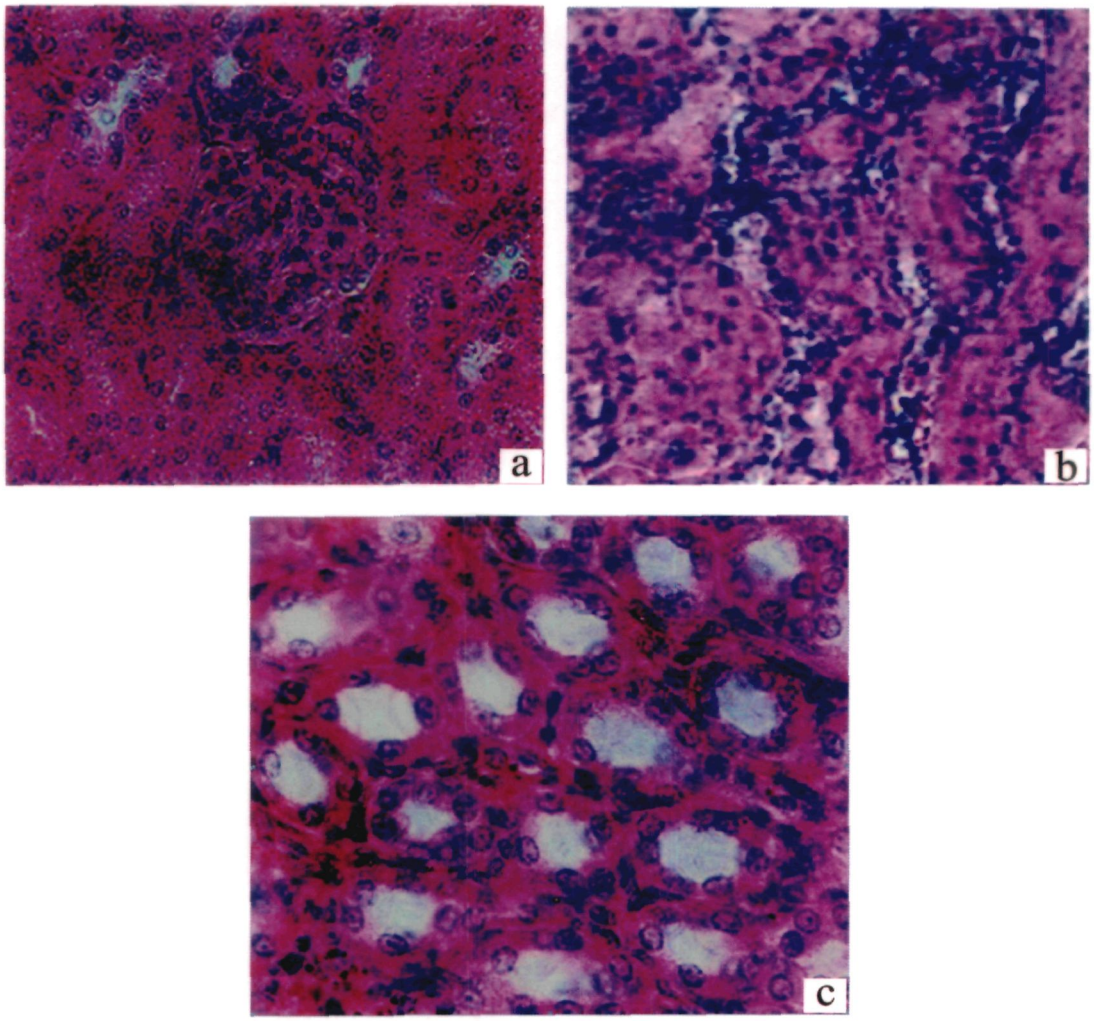


Figure 5. Sections of control mouse cortical kidney showing: (a), normal proximal convoluted tubules and a glomerulus (b), normal cortical region and (c), normal distal convoluted tubules. [(a: 40X), (b: 10X), (c: 40X) Haematoxylin and eosin]

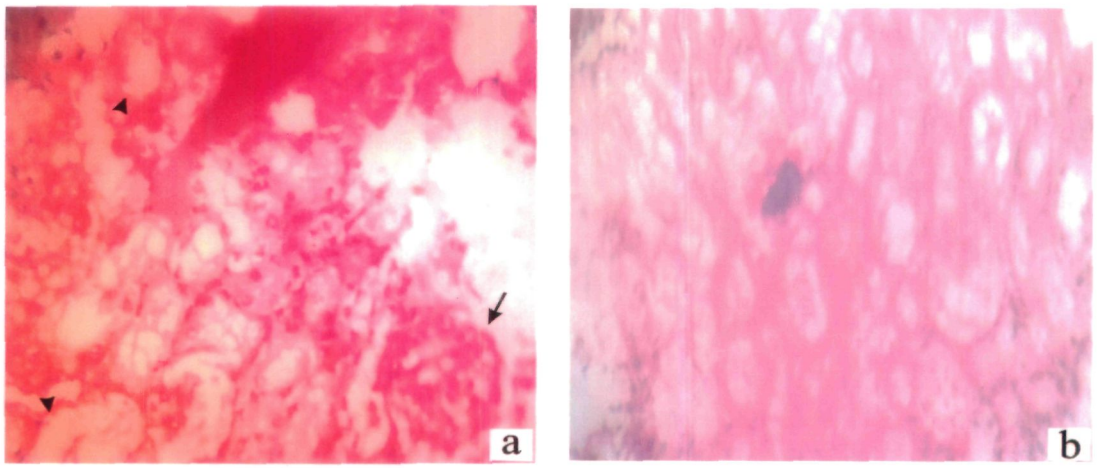


Figure 6. Sections of CPA positive control mouse cortical kidney showing: (a), moderate to severe glomerular oedema (arrow) and severe tubular degeneration and necrosis (arrow heads) at 24 hr and (b), severe tubular degeneration and necrosis at 48 hr. [(a, b: 40X) Haematoxylin and eosin]

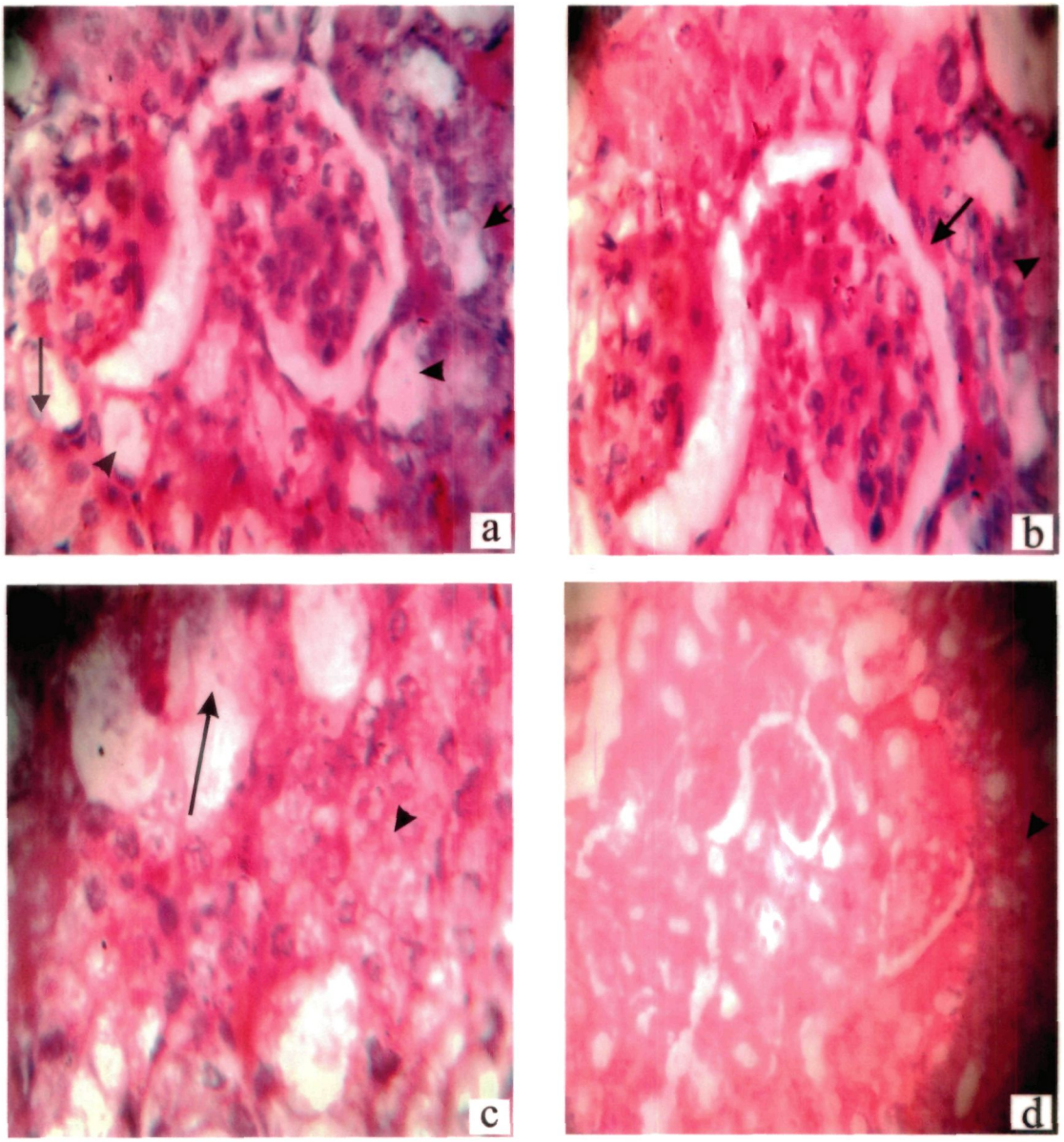


Figure 7. Sections of DDVP exposed (0.06 mg/kg bwt) mouse kidney showing: (a), mild degeneration and necrosis of proximal (dark arrow and arrow head) and distal (light arrow and arrowhead) convoluted tubules at 24 and 48 hr (b), apparently normal glomeruli (arrow) with only mild Interstitial Inflammation at 48 hr (arrowhead) (c), mild interstitial oedema at 24 and 48 hr (arrowhead) and scant tubular luminal eosinophilic debris at 48 hr (arrow) and (d), mild interstitial inflammation (arrowhead- inflammatory cell) at 48 hr. [(a, b, c: 40X), (d: 10X) Haematoxylin and eosin]

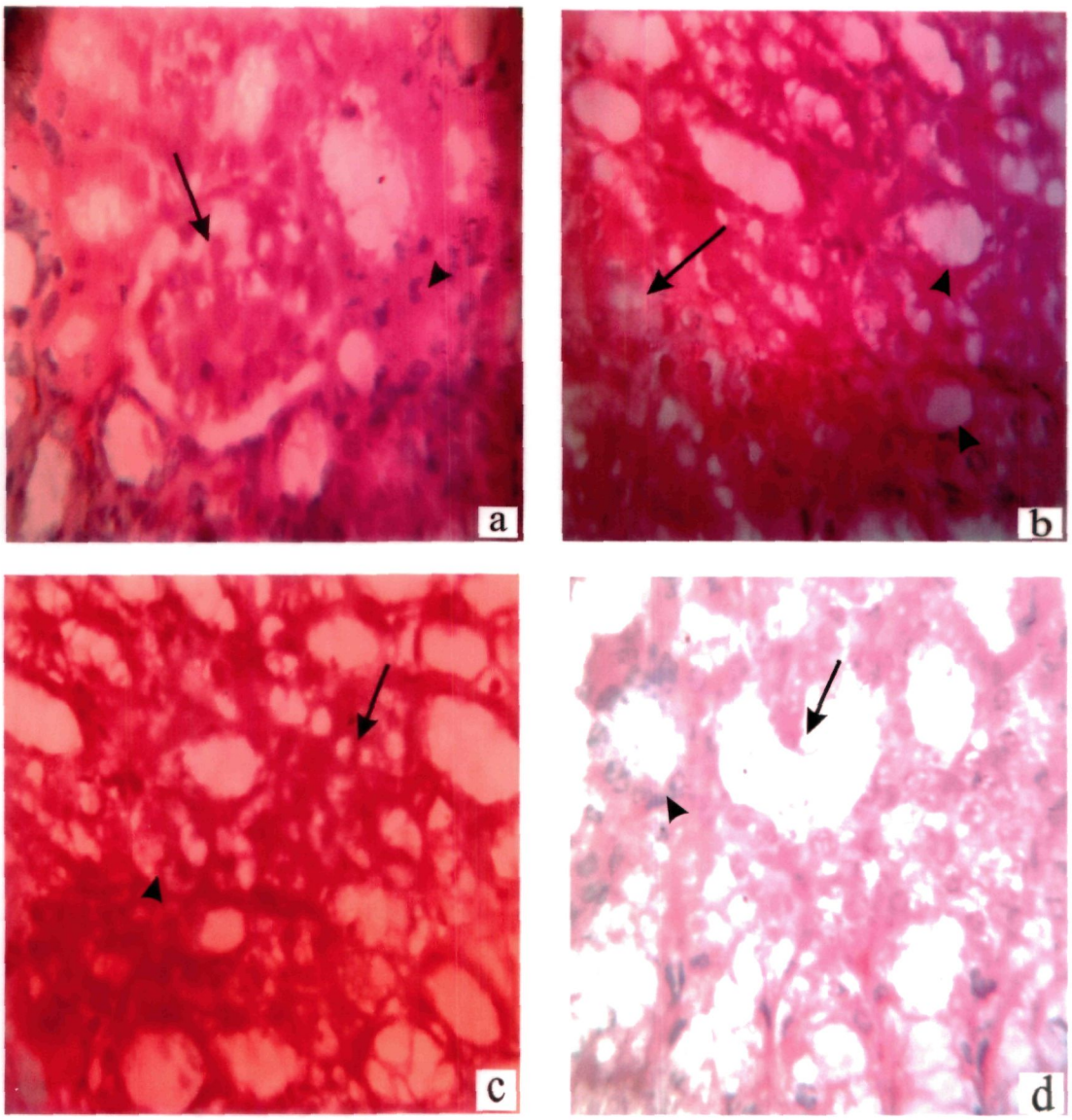


Figure 8. Sections of DDVP exposed (0.08 mg/kg bwt) mouse kidney showing: (a), mild glomerular oedema (arrow) with moderate Interstitial oedema (arrowhead) at 24 and 48 hr (b), moderate Proximal tubular (arrow) and distal tubular (arrowhead) degeneration and necrosis at 24 and 48 hr (c), moderate interstitial oedema (arrow), mild tubular regeneration (arrowhead) along with tubular degeneration and necrosis at 24 and 48 hr and (d), interstitial oedema, moderate tubular degeneration and necrosis along with moderate amount of tubular luminal interstitial debris 24 and 48 hr (arrow), mild to moderate tubular regeneration at 48 hr (arrowhead). [(a, b, c, d: 40X) Haematoxylin and eosin]

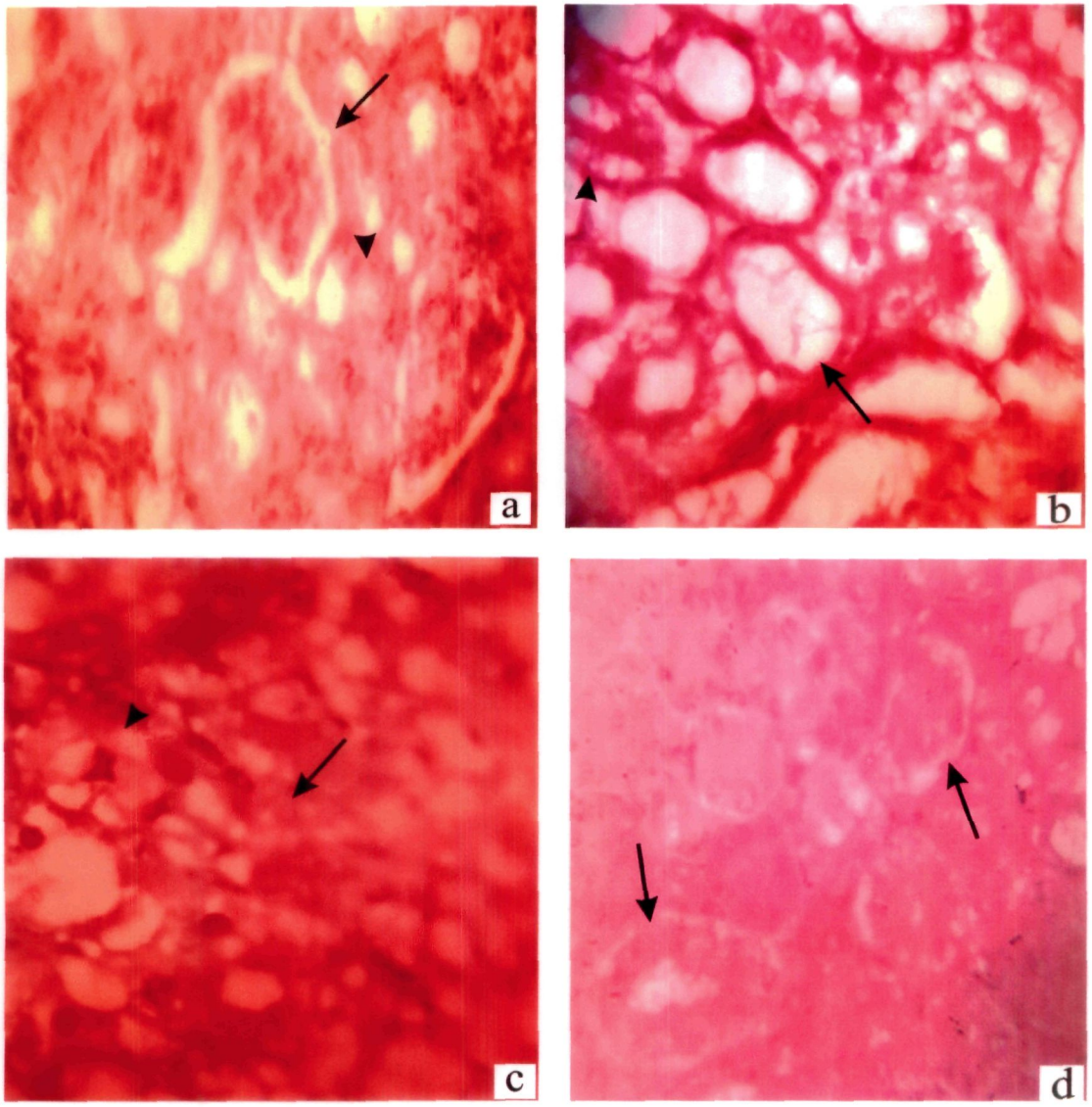


Figure 9. Sections of DDVP exposed (0.13 mg/kg bwt) mouse kidney showing: (a), moderate glomerular oedema (arrow), with moderate Interstitial oedema and moderate to severe tubular degeneration and atrophy (arrowhead) at 24 and 48 hr (b), severe tubular degeneration and atrophy (arrow), the proximal and distal tubules cannot be made out separately due to severe degenerative and atrophic changes, mild to moderate tubular regeneration (arrowhead) at 24 and 48 hr (c) severe tubular degeneration and necrosis, severe interstitial oedema (arrow) and significant amount of tubular interstitial eosinophilic debris (arrowhead) at 24 and 48 hr and (d), severe tubular degeneration and necrosis (arrow) at 24 and 48 hr. [(a, b, c, d: 40X) Haematoxylin and eosin]

All the toxicity signs were most prominent in animals exposed to 0.13 mg/kg bwt of the test chemical, however, these were also observed in the two lower exposed animals. The effect of DDVP was mainly severe on the tubular cells. Moreover, glomerular structure was also affected.

Kidney sections from the lowest dose dichlorophos groups at 72 hr, were comparable with controls, although TLECD were present in the cortex. Thus, histopathological examinations of the hematopoietic kidney indicates a dose – dependent injury in mice receiving ip single administration of dichlorophos (Table 9). Majority of the changes observed in our study, confirms that higher doses of DDVP applied intraperitoneally exert maximum renal injury. The toxicity symptoms showing less severe consequences as the time interval increased.

The degenerative changes observed in the present study is indicative of the action of dichlorophos metabolite on the hematopoietic cells of kidney, as they are the main sites of detoxification and excretion of toxic materials of administered agent.

- **Discussion**

In mice and rats, dichlorophos undergoes a quick hydrolysis and is excreted in the form of ionized products (Luty et al., 1998). Intensive metabolic activity and multiple functions account for kidney's sensitivity to the action of toxicants. Dichlorophos, including other OPIs, primarily being neurotoxic, inhibit acetylcholinesterase, which is responsible for enzymatic hydrolysis of acetylcholine in kidneys of rats (Ramesh et al., 1996). These insecticides (actual compound and metabolites also) are generally eliminated through urine (Koertz et al., 1997; Hughes et al., 1973; Sharp et al., 1972), but can also induce damage to the nephrons, as observed in our study. Hence, we found acute intraperitoneal administration of sub-lethal doses of Dichlorvos promote injuries to the PCTs, DCTs and glomeruli – showing the presence of tubular luminal eosinophilic cytoplasmic debris, interstitial inflammation, interstitial oedema and tubular regeneration. Under the influence of 0.13 mg/kg bwt dose, there is a prominent glomerular damage, tubular degeneration and necrosis, INIF, TLECD and IO. Organophosphate pesticides are known to be filtered at glomeruli (Nagano et al., 1992), and so actively secreted by tubular cells. A single acute dose of Dichlorophos in the present observations, promoted necrosis of renal tissue, thereby altering the normal morphology of nephrons. It can be inferred

that both glomerular structure as well as tubular cells of the hematopoietic kidney tissue are damaged in a Dichlorophos treated mouse.

The *in vivo* genotoxic assessment by way of toxicologic pathology is an integral component of evaluation of the toxicologic potential of pesticides, therapeutic agents and other chemicals. The results of histopathology obtained in the present study provide information regarding the overall genotoxic assessment, as well as, general metabolic and toxic processes, and target organ associated with exposure to dichlorophos. It will assist in establishing the mechanism of toxicity in diagnostic and experimental settings as suggested by Wanda et al. (2009). The present toxicologic pathology results provide valuable informations regarding effects on target organ effects, which can be compared within and across animal species. Thus, it is a strong predictor of the possible genotoxic response in the hematopoietic tissue of *Mus musculus* exposed to sub – lethal doses of dichlorophos.

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# CONCLUSIONS

# BIBLIOGRAPHY

## Conclusions

The cytotoxic and genotoxic potential explored using multiple assay system in *Mus musculus* by Dichlorophos - an organophosphorous compound. Multiple concentrations (0.06, 0.08 and 0.13 mg/kg bwt) and durations (24, 48 and 72 hrs) along with appropriate control; normal control (water) and for reference, a proved toxicant - Cyclophosphamide (CPA) as positive control were included, to facilitate relevant comparisons, within and outside the exposed groups. The specific aims were to measure cytotoxicity and genotoxicity targeting bone marrow hematopoietic cells, using endpoints - the micronuclei assessment in the mature and immature erythrocytes, quantification of cell proliferative index, in terms of mitotic indices and analysing the metaphase chromosomes for any induced structural aberrations. Extensive histopathological examinations of the hematopoietic renal cells were done to find cytogenetic damages. The injury or extent of damage in the two hematopoietic organs can be compared without doubt. The study also expected in identifying the probable target organ and this has been achieved effectively.

In the light of the stated aim and results obtained, we can conclude:

- In micronuclei assessment, initially erythrocytes - polychromatic, PCE and normochromatic, NCE, were clearly observed using differential staining technique, May - Gruenwald - Giemsa, on the basis of color. Observations were made with MN in both types. Many observations were noted MNPCE along with PCEs and NCEs lacking MN. The results of MN evaluation in bone marrow erythrocytes for each group per sacrifice interval were observed carefully and tabulated.

  The results demonstrated that the number of PCEs containing MN calculated separately for each dose could not significantly increase above the normal control frequencies but CPA registered significant difference with exposed - and increased MN found in dose - duration related manner.
- The ratio of PCE/NCE was not considerably affected in DDVP exposed, although a slight depression was seen as exposure time increased. This ratio significantly decreased in CPA positive controls. MNPCEs values

found significantly higher for all type of replicates in positive groups, against this DDVP exposed showing no significant increase.

- Dichlorophos induced MN induction in both PCEs and NCEs, but it was non - significant when compared to the normal control. Also, a change in the proportion of immature erythrocytes among total erythrocytes was recorded, an initial increase at 24 hr and the subsequent depression in 48, 72 hr was indicated to reflect the normal variability rather than bone marrow toxicity. The P/N ratio was also within the normal control range and not statistically significant.
- Quantification of cell proliferative index indicated that, MI was affected by the exposure of DDVP in mice but the MI proportions when compared to normal replicates, statistically very less difference at all the three increasing dose concentrations were recorded and thus, we can conclude that the MI (%) change in the normal and DDVP treated animals were almost comparable. Decreased MI is suggestive of lesser number of cells completing the cell cycle and hence, a high proportion of cells belonging to the resting stage of the cell cycle while the increase MI reflects increased cell proliferative activity. Dichlorophos treated animals showed no marked decrease or increase in the MI when contrasted with normal, reflecting DDVP neither inhibits nor induce mitotic progression.
- Metaphase chromosomes when analysed showed dichlorophos to affect the chromosomes at the metaphase stage. But the total percent aberration was in the range of the normal solvent control groups and hence, can be taken to lack statistical significance. The apparent changes observed in the cytogenetic parameters were assessed for reproducibility and biological significance. These results are not considered biologically meaningful since, the changes (increase or decrease) were not statistically significant compared to usual control.

Dichlorophos, as a compound / or its metabolite, do not elicit genotoxic response in bone marrow target cells, while confirmed to induce toxic response in the renal cells. Also, it is assured that the candidate chemical / or its metabolite reached the target organs, since both the tissue used in analysis were obtained from the same exposed group of *Mus musculus*. As a whole, when closely inspected, Dichlorophos - DDVP, reflected negative *in vivo* genotoxic potential in the hematopoietic bone marrow cells consistent with the negative cytogenetic results obtained in the presently, since it lack significant DNA

binding *in vivo*, in mice administered sub - lethal doses *via* ip injection. Due to the short chemical half - life of DDVP, the ip mode of administration in our case maximizes its rapid enzymatic degradation, minimizing the dose delivered to the target organ.

- Severe degenerative and pathological changes observed in the hematopoietic renal cells are observed at all doses and durations. The induced injury followed a dose dependent trend in the DDVP treated specimens. Major damages were induced in the tubules (both PCT and DCT) while noticeable glomerular oedema along with interstitial inflammation, tubular regeneration, tubular eosinophilic cytoplasmic debris and interstitial oedema.
- Nephrotoxicity of dichlorophos is confirmed, promoting severe altered renal architecture pertaining to the glomeruli, proximal and distal convoluted tubules and various other pathological changes. If taken clue from other studies, these injuries may be due to the intensive metabolic activity and multiple functions accounted for kidney's sensitivity to the action of toxicants and elimination of the actual compound and its metabolite through it, inducing damage to the hematopoietic renal cells and enzymatic hydrolysis of DDVP in kidney of mice.
- The value of the present *in vivo* genotoxic assessment is increased, relevant by the fact that genotoxic evaluation without obvious tissue restriction is recommended, wherever possible or required and, in the present case also, after confirmation of negative cytotoxicity results in bone marrow, another hematopoietic tissue - kidney was evaluated using a different and sensitive predictor in the overall toxic potential assessment of the chemical and a positive response was seen.
- Dichlorophos is a suspected carcinogen, since compounds negative in genotoxicity assessments may be carcinogen or non - carcinogen as cancer is triggered by genotoxic or non - genotoxic mechanism. Also, carcinogen with a non □ genotoxic mechanism may score negative and are then easily considered false negatives in genotoxicity tests whereas in fact, they are "correct" negatives in the specific tests. Also, a recent database by Kirkland et al., (2005) emphasizes the mechanism of action of carcinogenicity of 80% of the false negative substances to be non - genotoxic.

- Additional genotoxicity testing is recommended since dichlorophos is negative in the standard genotoxicity test battery but has shown severe toxicity in renal cells with insufficient evidence to establish a non-genotoxic mechanism.

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